The transcription factor CLAMP is required for neurogenesis in *Drosophila melanogaster*.

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Abstract

Neural stem cell (NSC) differentiation is controlled by cell-intrinsic and external signals from the stem cell niche including niche surface glia (SG). However, the mechanisms by which transcription factors drive NSC differentiation within the niche remain largely unknown. Here, we show that the transcription factor, Chromatin-linked adaptor for MSL proteins (CLAMP) is required for NSC differentiation. CLAMP promotes transcription of genes involved in stemness, proliferation, and glial development and represses transcription of genes involved in neurogenesis and niche survival. Consistent with transcriptional changes, CLAMP promotes NSC proliferation and SG production. Furthermore, glial-specific knock-down of *clamp* causes similar phenotypes to *clamp* null mutants. CLAMP motifs are present at many target genes including the glial-determining gene, *glial cells missing*, and *Notch*, a key regulator of neurogenesis. Collectively, our results suggest that CLAMP regulates a transcriptional program which drives NSC proliferation and differentiation *via* cell-intrinsic and niche-dependent mechanisms that involve niche glia.

Introduction

Brain development is an extremely complex yet carefully orchestrated process, regulated along spatiotemporal axes. For the formation of the functional brain circuitry to take place, neural stem cells (NSCs) must undergo multiple rounds of divisions and lineage decisions at each step of the process are governed by specific transcription factors (TFs)¹. In addition to the intrinsic developmental program, external metabolic and paracrine signals constitute another layer of developmental control over NSCs. Specifically, NSCs are part of a specialized microenvironment, the stem cell niche², which plays an architectural and nurturing role for the NSCs and their progeny. Moreover, the niche serves as a hub for the relay of metabolic and developmental signals that regulate differentiation of NSCs in order for the proper sequence of developmental events to be maintained³. In mammals, the stem cell niche is composed of various cell populations including neurons, glial cells, resident immune cells, the microglia, blood vessels which along with endothelial cells and glial cells form the blood-brain-barrier⁴. In *Drosophila*, the stem cell niche also consists of the NSCs, NSC progeny and glial cells of various types that are vital to the maintenance of the homeostatic balance required for proper neurogenesis to take place^{3,5-10}. However, little is understood about the gene regulatory mechanisms that generate the stem cell niche.

In *Drosophila melanogaster* neurogenesis occurs at two distinct stages: the embryonic and post-embryonic stages^{11,12}. The *Drosophila* central nervous system (CNS) is composed of the central brain (CB), the optic lobe (OL) and the ventral nerve cord (VNC). During the embryonic stage, CB neural stem cells (neuroblasts in *Drosophila*) proliferate to generate primary neurons until they become quiescent at the end of the embryonic stage. The optic lobe primordium which would form the OL, is generated by epithelial cells of the posterior procephalic region between embryonic stages 11-13¹³. Between stages 12-13 the OL primordium invaginates and attaches at the ventrolateral surface of the brain and embryonic OL neuroblasts (EONs) are formed¹⁴. EONs proliferate briefly to generate both glial and neuronal progeny and also enter

quiescence^{14,15}. Then during the early larval stage, concurrent with the initiation of larval feeding, metabolic signals impinge on the NSCs and cause exit from quiescence and initiation of the neurogenesis.

Neural stem cells divide asymmetrically, such that in each division they generate a new NSC and a daughter cell which will generate glial and/or neuronal progeny¹⁶. For the appropriate asymmetric division to take place, polarity determinants have to be sequestered on the apical and basal sides of the mother NSC: the apical complexes are inherited by the new NSC and maintain the proliferating state, whereas basal proteins are inherited by the daughter cell and will direct it to generate differentiated lineages. The Par protein complex which is apically localized also directs the localization of the basal determinants. Moreover, Par complex proteins such as the *Inscuteable* (Insc) and *Partner of Inscuteable* (*Pins*) regulate the orientation of the mitotic spindle which determines how the cleavage furrow will form during the division of the NSC^{17,18}.

In the *Drosophila* five distinct CNS glial types are identified based on morphological, positioning and molecular profile criteria: perineurial (PG), subperineurial glia (SPG), cortex glia (CG), astrocyte-like and ensheathing glia¹⁹. The first two types of glia constitute the *Drosophila* brain-blood barrier whereas PG, SPG and CG can also be classified as surface glia because they are closer to the brain surface¹⁵. Perineurial glia are found on the outside layer of the CNS and serve a nurturing role for the development and function of the NSCs and also regulate NSC proliferation *via* the secretion of Dally-like heparan sulfate proteoglycan (Dlp)⁵. Reciprocally, the proliferating NSCs secrete the BMP homologue, Glass bottom boat (Gbb) which acts to regulate their proliferation but also provides a survival signal to PG. This study also suggests that PG and NSCs have direct communication *via* direct membrane contact or transcytosis. SPG glia forms a tight protective layer of pleated septate junction contacts thus providing insulation of the nervous system from exogenous molecules. With the initiation of larval feeding, SPG and PG contribute to the reactivation of quiescent NSCs^{5,8,20} *via* fat body signaling and induction of insulin secretion from SPG. In conditions of oxidative stress and hypoxia, SPG and CG form lipid droplets (LDs) which protect NSCs from oxidative

stress^{6,21}. Cortex glia are especially interesting because these cells form a bespoke membrane chamber around diving NSCs and their progeny, thus providing not only protection and bidirectional signaling between the proliferating NSCs and their niche, but also serving as individual spatial units for each expanding NSC and its progeny³. A special subtype of CG, *mir8*⁺ surface-associated CG, controls neurogenesis in the OL^{13,19,22}. Thus, niche glia are important for the function of NSCs. However, the molecular mechanisms that direct the functional and nurturing relationship between niche glia and NSCs remain poorly understood.

Here we define a role for the essential CLAMP zinc finger TF²³ in neurogenesis which is partly mediated by niche glia. *Clamp* was discovered as part of a screen for factors that are necessary for the function of the dosage compensation machinery in *Drosophila melanogaster* males²⁴. In contrast to the MSL complex components, CLAMP is essential for viability of both males and females and has transcriptional targets on all chromosomes, albeit with a stronger binding affinity on the X^{23,25}. Since its first characterization as a required link for the MSL complex to bind to the X-chromosome^{26,27}, CLAMP is emerging as a multifaceted transcription factor with roles including chromatin organization²⁸, histone locus function²⁹ and regulation of Pol II function³⁰. The identification of *Clamp* (CG1832), as a functional interactor of *embryonic lethal abnormal visual system (elav)* and *erect wings (ewg)*, critical regulators of embryonic and post-embryonic neural development, hinted at a neuronal role of CLAMP^{31,32}. Therefore, we hypothesized that CLAMP is a TF which regulates neuronal development and NSC biology.

To test our hypothesis, we investigated the role of CLAMP in nervous system development. We find that CLAMP is important both for neuronal and glial cell generation and regulates NSC proliferation *via* cell-intrinsic and niche-dependent mechanisms. We used a CRISPR/Cas9-generated frameshift mutant in the *clamp* gene which eliminates the expression of the CLAMP protein²⁵ to determine that lack of CLAMP results in defects in brain development. Specifically, *clamp* null mutants have a smaller brain than controls, with the most prominent defect observed in the optic lobe which is almost entirely lost. Our mRNA-

sequencing data from *clamp* mutant embryos and larval brains followed by temporal gene ontology (GO) analysis determined that CLAMP positively regulates stemness/NSC proliferation genes and glial-development genes. Binding motif analysis revealed the CLAMP motifs are present at key neurogenesis genes such as *bazooka*, *gbb* and the glial-development regulator, *glial cells missing*. We validated transcriptomics by using immunostaining to show that *clamp* null mutants exhibit a brain-wide reduction in proliferating NSCs and surface glia and a profound defect in the development of the OL. Knock-down of *clamp* in glia but not in neurons, revealed that NSC proliferation and glia generation are regulated in part via glial *clamp*. Our results support a model in which CLAMP promotes the precise timing of NSC differentiation by regulating genes which drive intrinsic and niche-glial mechanisms.

Results

CLAMP is ubiquitously expressed throughout the L3 nervous system and is present in proliferating neuroblasts.

Clamp is maternally deposited and therefore *clamp* transcript and protein levels²⁹ are present at high levels during embryogenesis. Near the end of embryogenesis (16-18 hours) during tissue differentiation and the cessation of the first wave of neurogenesis, *clamp* transcript levels show a reduction followed by a sharp increase between 20-24 hours and then a decrease throughout the post-embryonic neurogenesis period (Fig. 1a). Tissue-specific expression data from modENCODE Development RNA-Seq Collection^{33,34}/Flybase³⁵ show that *clamp* is highly expressed in the L3 central nervous system (Fig. 1a). To define the role of CLAMP in neural development, we mapped its expression in the developing L3 nervous system. Immunofluorescence for CLAMP in L3 larvae revealed that it is ubiquitously expressed in the nucleus throughout the larval nervous system, both in cells of the brain and the ventral nerve cord (VNC), (Figs. 1c-g and h). Interestingly, a subset of processes in the VNC also show protein localization of CLAMP,

suggesting that it may be transported to neuronal arborizations (Fig. 1c, axonal processes indicated by arrows). Co-staining with the neural stem cell (NSC) marker Deadpan (Dpn), revealed that CLAMP is expressed in NSCs (Figs.1i, j). Interestingly, CLAMP maintains its expression in mitotic/proliferating NSCs, as seen by co-staining of Dpn and PCNA, both in the central brain and the OL (Figs.1i, j). The distribution of the TF PCNA³⁶ within the cell nucleus changes during the progression of the cell cycle allowing its nuclear localization to mark distinct phases of the cell-cycle^{37,38}. In this way, we determined that CLAMP is present in S, G1 and G2-phase cells (Fig.1j).

Lack of CLAMP leads to deficits in larval development

To determine the role of CLAMP in nervous system development, we characterized brain development in *clamp*² mutants which we previously generated in our lab *via* the CRISPR/Cas9 approach²⁵. Specifically, in these mutants, a frameshift mutation in the *clamp* gene abolishes expression of CLAMP protein²⁵ (from here on referred as *clamp* null). Due to the requirement for CLAMP for the recruitment of the dosage compensation complex in males²³, the majority of male larvae die at the L2 stage. In contrast, female larvae die at the late L3/prepupa stage²⁵. Because we are interested in the essential role of CLAMP outside of dosage compensation and due to the small number of male *clamp* null larvae, we only used female larvae and focused on the L3 stage when larval brains can be dissected. Additionally, we matched control animals for developmental stage with the *clamp* null larvae (see Materials and methods for details). Comparison of *clamp* null L3 larvae to controls revealed that they are significantly smaller in size than matched controls (Figs.2a and b). Furthermore, macroscopic examination of the central nervous system of *clamp* null larvae revealed that their brains are significantly smaller compared to controls (Figs.2c and d). Western blotting for CLAMP in *clamp* null larvae brains verified the absence of CLAMP protein (Fig.2e).

Lack of CLAMP causes defects in brain development

In order to evaluate CNS development in the *clamp null* mutants, for the majority of our experiments we focused on the L3 stage and specifically the mid/late L3 stage (~120 hours after larval hatching (ALH)), because the *clamp* mutants die during late L3/early pupa stage. Additionally, to better understand the extent of neurodevelopmental defects in the *clamp null* animals, we also examined early L3 larvae brains. The overall morphology of the CNS, as observed with DAPI staining, is different between nulls and controls as described below (Figs.3a and e). As neural development progresses through the larval stages, the neuroepithelial (NE) cells of the optic lobe (OL) start to differentiate into neuroblasts (NBs), which will subsequently divide to give rise to neurons and glia¹⁵. We observed that the *clamp null* OL is profoundly reduced in size. The *clamp null* OL neuroepithelia at 120h ALH resemble the 72h ALH control neuroepithelia (i.e., early L3), indicating a likely arrest of the NE optic lobe cells (Figs. 3a, b, e and f). Similarly, the *clamp* mutant central brain (CB) of the mid/late L3, has an underdeveloped morphology and is more similar to the early CB of the control (Figs.3f and a).

Next, we examined NSCs in *clamp null* and control brains. Full z-stacks of the Dpn-positive NSCs both in the early and mid/late L3 stages (Figs. 3c, d, g and h), revealed that in the *clamp null* NB development both in the CB and OL, is considerably delayed from the early L3 stage and remains so until the mid/late L3 stage when the animals die. The OL NB population is greatly affected as the Dpn-positive area at the mid/late L3 stage is much smaller than in controls (Figs 3d and h, dashed outlines). These observations suggest that the deficits observed in brain formation during the L3 stage of the *clamp null* are may be initiated at even earlier developmental stages leading to a dramatic defect in OL development. Interestingly, comparison of early L3 *clamp* heterozygotes (*clamp*^{+/+}) and early L3 *clamp* nulls indicates that a single copy of the *clamp* gene is sufficient to maintain the overall morphology of the developing OL (Supplementary. Fig. 1).

Developmental transcriptomic analysis of clamp null mutants reveals a role for CLAMP in nervous system development

The neurodevelopmental deficits observed in the larval *clamp* null mutant brain suggest that the developmental action of CLAMP might initiate at an even earlier developmental stage. As a ubiquitously-expressed TF, CLAMP is present throughout the nervous system from the early embryonic stages due to the deposition of maternal transcripts into the egg³⁹ (Fig.1a). Each neurodevelopmental stage is characterized by specific transcriptomic events which are controlled by specific TF factor networks¹. Thus, in order to understand how CLAMP regulates nervous system development on the transcriptomic level, we performed a developmental RNA-sequencing experiment in the *clamp* null mutant during the following key developmental stages: A) the late embryonic stage (16-18 hours), a time point where embryonic neurogenesis pauses¹⁴ and most NBs enter quiescence; and B) larval brains dissected from the mid/late L3 stage during which nervous system development in terms of neuronal and glial cell production are at their maximum (see Fig.4a)⁴⁰. Additionally, selection of the specific embryonic time point was based on studies by Haussmann et al. (2008, 2010), where *clamp* was identified as a functional interactor of *elav* and *ewg*, in 16-18h embryos^{32, 31}.

GO analysis revealed statistically significant enrichment for developmental/stem cell-related GO categories, including neural development, at both the embryonic and larval developmental stages (Fig.4a). Due to the neurodevelopmental defects in the *clamp* null mutant, we then specifically mined our expression data for differential expression of genes (DEGs) that are implicated in cell cycle attributes, nervous system development and neural stem cell biology. Additionally, we observed enrichment for GO categories involving metabolic pathways. Because post-embryonic neurogenesis is largely orchestrated by signals originating from nutrient availability and insulin signaling^{6,41,42}, we decided to also mine for metabolism-related DEGs (Fig.4a).

To evaluate the possibility of direct regulation of DEGs by CLAMP, we determined where CLAMP binding sites are present using FIMO analysis⁴³. Previously characterized CLAMP binding motifs were used for this analysis⁴⁴. Interestingly, genes that contain putative CLAMP binding sites and are common between the embryonic and L3 stages include the important proliferation/mitosis regulators *bazooka (baz)*, *separation anxiety (san)*, a N-terminal acetyltransferase, required for mitotic chromosome segregation⁴⁵, *cyclin dependent kinase 7 (cdk7)*, vital for mitosis⁴⁶, *PCNA* and *Notch*; *sobremesa (sbm)* an SLC7 amino acid transporter expressed in glia and regulating brain development via ecdysone signaling⁴¹; and *Negative elongation factor E (nelf-E)* (Fig.4e, Embryo and L3 panel).

Developmental stage-specific genes that contain putative CLAMP binding sites include: A) Embryo stage: *anaphase promoting complex subunit 7 (APC7)*; *pontin (pont)* which is part of the TIP60 complex which regulates NSC attributes; *aurora A (aurA)* a kinase regulating asymmetric protein localization and thus required for NSC proliferation⁴⁷; *extra sex combs (esc)*, a component of the PRC2 complex which regulates NSC proliferation and neural development⁴⁸ and *fruitless (fru)*, implicated in the formation of axonal projections in the embryonic CNS⁴⁹ (Fig.4e, Embryo panel). B) L3 stage: Even more interesting during the L3 stage (Fig.4e, L3 panel), CLAMP binding motifs are found in *gcm*, an essential regulator of glial development; *glass bottom boat (gbb)* which regulates both NB proliferation and surface glia survival; *tailless (tll)*, a key regulator of optic lobe development and *anaplastic lymphoma kinase (Alk)* which is implicated in cell cycle regulation and NSC proliferation; and *Dichaete (D)*, a TF regulating neuronal fate in the OL.

Therefore, CLAMP may be exerting direct control on neural development by affecting the transcription of key players that function in multiple processes including asymmetric cell division, cell cycle and hormone signaling. CLAMP may be directly regulating genes controlling different aspects of neural development in different cell types such a glia (*gcm*: gliogenesis) or NSCs/neuroblasts (*gbb*: expressed in NSCs to control proliferation and SG survival⁵). Therefore, the neurodevelopmental phenotypes observed in the *clamp* null

mutant might result from combinatorial changes in diverse signaling cascades originating from different cell types.

We then proceeded to analyze the individual datasets which revealed that in the *clamp* null embryo, 5917 genes were differentially regulated ($\text{padj} \leq 0.05$, Fig.4b and Supplementary Table 1a). Functional analysis of the DEGs revealed highly statistically-significant enrichment in categories such as cell cycle regulation, cell cycle checkpoints, mitotic cell cycle phase transition, neurogenesis and nervous system development, metabolic processes, synaptic development and signaling (Supplementary Table 1b). We focused our analysis on two major highly enriched GO categories: i). *Cell Cycle Regulation* [GO:0051726, padj : 6.22E-11] and ii). *Nervous System Development* [GO:0007399, padj : 4.32261E-05], due to the fact we identified key genes implicated in both categories as putative direct targets of CLAMP. Specifically, these are genes directly regulating NSC proliferation and division mode: *aurora A (aurA)*, *bazooka (baz)*, *pcna* and *notch* (Fig.4E).

For the *GO:0051726 -Cell Cycle Regulation* category (Supplementary Table 1c), functional analysis of the participating DEGs revealed specific roles including in G2/M transition of mitotic cell cycle, regulation of chromosome segregation, cell cycle G1/S phase transition, negative regulation of nervous system development and of insulin signaling (Fig.4c). These results are in line with CLAMP having direct control over the expression of genes implicated in these processes including, *sbm* (Fig.4E) which is implicated in regulation of neurogenesis through hormone signaling.

Expression of *elav*, an RNA-binding protein and master regulator of neural development, was increased [L2FC: 0.44; padj : 0.045]. *elav* encodes a protein that regulates the expression of *ewg*. Both *elav* and *ewg* are critical for nervous system development, and neuronal maturation⁵⁰. Hausmann et al. (2008, 2010)^{31,32} reported that *clamp* along with a group of nine functionally-related genes which includes *groucho*, another critical developmental regulator⁵¹, are positively regulated by EWG and restrict synaptic growth (Table 1).

Analysis of the Haussman et al. (2008) DEGs in the *clamp* null embryo mRNA-seq data (16-18 hrs), revealed that 9 out of 10 DEGs regulated by *ewg* are also DEGs in the *clamp* null embryos. Furthermore, the synaptic function-related GO categories were highly enriched in the *clamp* null embryos, as shown for the functionally-related genes that are regulated by *elav* and *ewg*^{31,32}. Therefore, it is possible that a synergistic relationship between the CLAMP and the ELAV/EWG pathways exists, in which they regulate each other's expression to assure that brain development occurs normally.

Meta-analysis of the GO:0007399: Nervous System development category (Supplementary Table 1d), revealed strong enrichment in the sub-categories of neurogenesis and central nervous system, visual system development and glial cell development and differentiation (Fig.4d). Some of the DEGs that are part of the *Nervous System development* GO category include: 1) *polo*, *aPKC*, *par6*, *pins*, *aurA* and *baz* components of the Par-complex which regulates asymmetric neuroblast divisions; 2) *PCNA*, *RFC38*, *dacapo*, and *Reptin* (part of the Tip60 complex), which participate in the molecular network controlling cell polarity, asymmetric NSC division and cell fate⁵²; 3) *CSN1b*, *CSN4* and *non-stop* implicated in lamina glia cell migration and optic lobe development⁵³. Importantly, *baz* and *pcna* are both candidate direct targets of CLAMP as seen by binding motif analysis (Fig.4E), thus its is highly possible that the enrichment in these categories is the result of direct effects of CLAMP in these key loci.

Furthermore, several DEGs critical for neural development and NSC biology were identified: *Notch* [L2FC: -0.5, padj: 0.01], *lola* [L2FC: -0.6, padj: 0.004], and components of the Brahma chromatin remodeling complex such as *moira* [L2FC: -1.019, padj: 6.21E-05]^{52,54,55}. Importantly, many DEGs are part of specific TF-networks that regulate different aspects of neural stem cell renewal identified by Neumuller et al. (2011): *Su(z)12*, *Su(var)2-10*, the Hdac1 *Rpd3*, and *lola*. Notch signaling is critical for the balance between proliferation and differentiation in NSCs and it has been suggested that *lola* can have distinct effects on the Notch pathway depending on the expressed *lola* isoform⁵². Moreover, Notch controls neuroblast selection

and delamination⁵⁶ and is a key regulator of asymmetric divisions throughout the nervous system^{57,58}. Additionally, Notch maintains the balance between symmetrically and asymmetrically dividing stem cell pools in the optic lobe⁵⁹. Importantly, we have shown that *Notch* may be under direct control by CLAMP (Fig.4E), thus transcriptome changes associated with Notch signaling may be results of the CLAMP-*notch* interaction.

Categorization of the embryonic DEGs allowed us to identify potential pathways in which CLAMP is likely to regulate target genes. Overall, lack of CLAMP results in dysregulation of three inter-dependent gene/TF networks: 1) neurogenesis/gliogenesis; 2) cell cycle/asymmetric division; and 3) neuronal arborization/synaptogenesis.

Transcriptomic analysis of overlapping embryo and L3 *clamp* null DEGs implicates CLAMP in regulating embryonic and post-embryonic neurogenesis.

The transition from the embryonic to the post-embryonic stage is accompanied by many physiological changes with the most notable being the introduction of feeding which brings about a cascade of metabolic signaling that induces activation of quiescent NSCs and neurogenesis. Comparison of the *clamp* null embryo and L3 mRNA-seq results revealed that a large number of DEGs were shared amongst the two stages (2363 DEGs). (Fig.4b). Meta-analysis of the top two GO categories revealed that the participating genes have roles in nervous system development and neurogenesis, mitotic cell cycle, G protein-coupled receptor signaling pathway and neurotransmitter biosynthesis suggesting a continuum in the roles that CLAMP has in neural development pre- and post-embryonically. These results are in line with the fact that key genes implicated in the above categories are putative direct targets of CLAMP, especially, *baz*, *pcna* and *notch* and are consistent with the deficits observed in L3 brain development (Figs. 2 and 3).

The fact that cell cycle-related categories were enriched in our transcriptomic analysis is interesting because CLAMP maintains its expression in L3 proliferating NSCs and co-localizes with PCNA (Figs.1i andj), which has critical roles in the cell cycle³⁸ and is a candidate direct target of CLAMP. The expression of *pcna* is downregulated in both developmental stages (*clamp* null embryo L2FC: -1.692 and L3 larva L2FC: -0.824, Supplementary Tables 1a and 2a) in addition to cell cycle regulators/chromosome dynamics factors such as *AurB*, *atms*, *baz*, *Cdc16*, suggesting that CLAMP regulates cell cycle progression and dynamics directly (Fig.4E) .

Clamp is maternally deposited and its expression is high during the majority of embryogenesis²⁹ (Fig.1a). While the persistence of maternal *clamp* was not specifically evaluated in late embryos, a study from our lab showed that *clamp* deposited by *clamp* heterozygous mothers persists beyond the embryonic stage and is present in the histone locus body throughout the development of the larval salivary gland ²⁹. Thus, it is possible that a trace amount of maternally deposited *clamp* could still be present at 16-18 hr embryos.

CLAMP regulates genes controlling NSC attributes, glia development and function

The L3 stage encompasses a dramatic shift in the establishment of the nervous system. A plethora of signaling pathways, including metabolic signals stimulated by larval feeding^{60,42,61}, are integrated at the L3 stage to allow NSCs to promote maturation of the nervous system. Consistent with this shift in NSC function, GO analysis of the L3 DEGs (Supplementary Table 2b) revealed an enrichment in several broad categories including: neurogenesis, mitotic DNA replication, and neuroblast differentiation. We find changes in many genes critical for nervous system development including those in the following categories: 1) neuroblast attributes; 2) cell cycle dynamics; 3) glial cell development; 4) optic lobe development; 5) ecdysone & insulin signaling dynamics; 6) lipid metabolism; 7) Clamp-related interactors; 8) Synapse function & structure.

Next, we classified DEGs as down-regulated or upregulated in the *clamp* null mutant compared with wild type controls (downregulated vs. upregulated, Tables 2 and 3, respectively). Downregulated genes are genes that are putatively activated by CLAMP in wild type animals. These genes can be classified into the following categories: 1) *NSC attributes* (cell-cycle progression, cell polarity, and survival); 2) *control of NSC evolution/differentiation*; 3) *regulation of gliogenesis*; 4) *optic lobe development-specific*. In contrast, upregulated genes that are normally repressed by CLAMP are part of the following categories: 1) *lipid metabolism/promoting mobilization of lipids*; 2) *ecdysone and insulin signaling in glia cells*; 3) *pro-arborization/synapse formation and function*; 4) *pro-neural/suppression of proliferation*. Based on these data, we hypothesized that loss of *clamp* leads to deficits in neural stem cell attributes and promotes changes that lead to the precocious acquisition of a neuronal fate at the expense of gliogenesis. Our expression data also suggest that loss of CLAMP may cause concomitant activation of lipid metabolism/mobilization as a compensatory response from the stem cell-niche. Additionally, we observed strong downregulation in genes orchestrating OL development such as *eya*, *tll*, *dac*, *optix* (See Table 2 and Supplementary Tables 2d), of which *tll* is a putative direct target of CLAMP (Fig.4E). These results are consistent with the greatly reduced size of the L3 OL (Fig.3) and suggest a greater sensitivity of OL NSCs to the absence of CLAMP compared with the rest of the brain.

CLAMP promotes NSC proliferation and prevents precocious neurogenesis

Our developmental transcriptomic analysis lead us to hypothesize that CLAMP regulates neural stem cell/neuroblast attributes, progeny fate and synaptic circuit formation (axonogenesis, synaptogenesis). For example, lack of CLAMP leads to a downregulation of genes that regulate neural stem cell stemness and cell cycle progression both in embryos and larvae (Figs.4c-e). Therefore, we hypothesized that lack of CLAMP promotes precocious neurogenesis at the expense of glia formation through the L3 stage. To test our hypothesis that CLAMP regulates neural stem cell proliferation, we evaluated mid-late L3 brains for proliferating NSCs using the S-phase marker EdU and the NSC marker Dpn. In agreement with our initial

observations (Fig.3), the OL in *clamp* null larvae brains was dramatically reduced in size compared to controls, and both the CB and OL contained fewer proliferating NSCs (EdU⁺Dpn⁺, Figs.5a and c and Supplementary Figs.2 a, b). Quantification of total proliferating NSCs throughout the CNS yielded a dramatic ~90% reduction compared to controls (Fig. 5e). Moreover, the total number of proliferating cells was reduced compared to controls, suggesting a reduced proliferation of stem cells and/or change in cell cycle dynamics in the absence of CLAMP (EdU+ Fig.5e).

Genes encoded by many pro-neural TFs are upregulated in the absence of CLAMP (Table 3), in addition to TFs that promote cell cycle regulation in proliferating stem cells. Specifically, in the L3 *clamp* null brain the *Dpn* transcript, which regulates NSC proliferation, was significantly reduced (Table 2; L2FC: -0.665, padj: 0.00245). Reduction in *Dpn* transcript levels correlates with the reduced numbers of proliferating NSCs in the *clamp* null and suggests that neuron production may be altered in the absence of *clamp*. The TF Prospero (Pros), acts as a master regulator driving the decision of stem cells to either self-renew or differentiate⁶², while nuclear presence of Pros signals the initiation of neuronal differentiation^{62,63}. Thus, to evaluate changes in neuronal production in the *clamp* null brain we performed Pros staining. We found that the *clamp* null brain contained more clusters of nuclear Pros than matched controls, thus indicating a rapid change in NSC cell fate attributes⁶⁴ (Figs.5b and d, arrows indicate Pros⁺ clusters). Interestingly, while the total number of NSCs in the *clamp* null brain was reduced by approximately 70% (Dpn⁺, Fig.5f), Pros⁺ cell numbers were increased by approximately 70% (Pros⁺, Fig.5f), indicating that stem cells are acquiring a precocious neuronal fate. The reduction in NSCs is particularly evident in the OL, where very few Dpn⁺ cells were observed and instead many Pros⁺ cells were present (Figs.5a and c- dashed lines and outlines in Figs.5b and d indicate the OL). Pros, acts a transcriptional repressor of self-renewal genes and induces differentiation⁴⁷. In the *clamp* null brains, a significantly lower number of cycling stem cells have nuclear Pros (Pros+EdU⁺, Sup.Fig.2c), suggesting that in the absence of CLAMP, neural stem cells either undergo fewer cell division cycles and/or exit the cell cycle to acquire a precocious differentiated fate.

To further evaluate changes in the neuronal pool we performed Elav staining to label neurons⁶⁵ which revealed that *clamp* null brains contained a higher density of Elav⁺ clusters compared to controls (Figs. 5g and h). However, even though the overall Elav⁺ cluster density increased the Elav⁺ zone in the OL was reduced compared to controls (Figs. 5g and h, yellow outlines). Immunostaining with the apoptotic marker Dcp1 revealed increased and specific presence of Dcp1⁺ nuclei in the OL in *clamp* nulls, compared to the controls which instead had more widespread apoptosis (Figs. 5i and j). Moreover, in more dorsal optical sections big Dcp1⁺ clusters were visible in the medulla and lamina area (Supplementary Figs. 3a and b). These results suggest that in the absence of CLAMP a pro-differentiation pathway is activated in stem cells at the expense of their self-renewal which especially impacts the OL.

Lack of CLAMP results in reduction in number of surface glia and increased lipid droplet concentration

OL development is highly dependent on glia-neuron interactions because glia form a supportive niche for neural stem cells to function^{13,66}. Our transcriptomic analysis supports the hypothesis that lack of CLAMP promotes neuronal differentiation at the expense of gliogenesis from the late embryo stage. Furthermore, the TF master regulator of gliogenesis, *gcm*, is a putative CLAMP target (Fig. 4e).

Therefore, the defects observed in OL development of the L3 *clamp* null are likely to be the combinatorial result of deficits in glia formation and precocious neurogenesis. It was recently shown that surface glia (SG) are required for NB proliferation⁶⁷ using two different mechanisms: 1) SG regulate NSC proliferation via the secretion of the proteoglycan Dally-like, (Dlp)⁵; 2) Stem cells secrete the BMP-ligand Glass bottom boat (Gbb) which regulates both their proliferation and survival. Furthermore, *gbb* is a putative direct transcriptional target for CLAMP (Fig. 4e) because it contains CLAMP binding sites and is downregulated in the *clamp* null L3 brains (Supplementary Table 2d). Also, we observed enrichment of the BMP-signaling GO category in the *clamp* null (Fig. 4d). Therefore, it is also possible that the effects we see on neural development in the *clamp* null brains are the result of changes in the dynamics between SG and NSCs. To

investigate this possibility, we used the glia-specific marker Repo to mark glial cells and we counted SG in L3 brains (Ref Avet-Rochex)⁴⁰. This approach revealed that the SG number in the *clamp* null was reduced by ~25 % compared to the control (Figs.6 a-d). In addition to changes in SG, we observed that the distribution and numbers of glia throughout the brain and especially in the OL exhibited a different morphology within the lobula plug (Supplementary Figs. 3c-f). Overall, these data suggest that CLAMP specifically regulates gliogenesis and glia distribution especially in the OL.

To determine if the effect on SG numbers was due to the lack of CLAMP in glia specifically, we used the GAL4-UAS system to induce *clamp*-RNAi knock down (KD) using the glia-specific driver, *repo-GAL4*⁶⁸. Interestingly, we find that in the *repo>clamp RNAi* brains, the number of SG was reduced to a similar extent as in the *clamp* nulls (Repo+, Figs. 6a-d). To determine whether glia-specific or neuronal-specific *clamp* depletion has differential effects on the proliferation (EdU+) and the number of NSCs (Dpn+ cells), we also performed neuronal *clamp* KD by crossing to an *elav-GAL4* fly line⁶⁹. Comparison with the data we already obtained from the *clamp* null and control brains (see Figs.5e and f) revealed that *clamp* KD in neuronal lineages did not result in significant changes either in EdU+ or Dpn+ cells, glia-specific *clamp* KD caused a reduction in the number of total proliferating cells (EdU+). Therefore, glial CLAMP regulates proliferation of NSCs likely *via* both cell autonomous mechanisms (e.g., reduction in Dpn+ gliogenic NSCs) and *via* non-cell autonomous mechanisms (regulation of proliferation by niche glia).

Lack of CLAMP causes an imbalance in NSC niche homeostasis

One mechanism by which the NSC niche protects the stem cells from environmental stressors like hypoxia and oxidative stress is by the mobilization of polyunsaturated fatty acids away from lipid membranes and into lipid droplets (LDs) formed by cortex and subperineural glia cells (SPG). The GO analysis of the L3 *clamp* null transcriptomic data (Supplementary Table 2b) showed that the category metabolic process (GO:0008152) was amongst the most enriched, and genes implicated in lipid metabolism were significantly

upregulated (Sup. Table 2c). Additionally, both *sbm* and *wunen* (*wun*), genes regulating neural development via hormone signaling and regulation of lipid levels respectively, are both putative direct targets of CLAMP (Fig. 4E) which are upregulated in the L3 *clamp* null brains (Table 3). Therefore, we hypothesized that in the absence of CLAMP, the niche lipid droplet formation mechanism is activated.

To test this hypothesis, we quantified LDs following staining with the neutral lipid-specific dye LipidTOX. We found that the concentration of LDs was increased by 50% in the *clamp* null brain compared to controls (Figs. 6j-p). Also, the distribution of LDs in the *clamp* null brain differed compared to control brains. Interestingly, large LD formations were observed surrounding dividing NBs in the *clamp* null more often than in the control brain (Figs. 6l and m and Supplementary Fig. 4). Collectively these results suggest that despite the lack of CLAMP causing a reduction in LD-producing SG, the remaining SG cells undergo lipid mobilization and increase LD formation, perhaps in an attempt to save the remaining proliferating NSCs.

In recent years, LD biology and function has been gaining traction in the context of both basic biology and disease because LDs have proven to be critical and plastic organelles that actively participate in various cellular functions⁷⁰. An intriguing observation is the function of LDs as a storage location for free histones, which in turn affects mitotic chromosome structure. Specifically, interactions between the LD-surface-located Invadolisin and the SAGA chromatin modifying complex regulate chromosome structure⁷¹⁻⁷³. The GO analysis of the L3 stage transcriptomic data indicated a deficit in the neuroblast division mechanism and in chromosomal organization (See Supplementary Table 2b and Table 2). Additionally, critical regulators of asymmetric NSC division and mitotic spindle orientation were significantly down-regulated in the *clamp* null brain (see Table 2) and some were identified as putative CLAMP targets both at the embryo and L3 stage (Fig. 4e).

Based on the connection between LDs and chromosome behavior and the results from our *clamp* null L3 RNA-seq, we decided to check for morphological differences in mitotic neuroblast chromosomes from

clamp null brains. Interestingly in mitotic chromosome preparations from *clamp* null brains, we observed hyper-condensed, “snowflake-like” chromosome formations in contrast to the elongated and well separated chromosomes observed in control preparations (Figs.7a-d, yellow outlines). Occasionally, in the *clamp* null preparations we would observe chromosomes with normal morphology, but this was less common (Fig.7d, magenta outline). Co-staining with the differentiation marker Pros, also revealed the presence of more nuclei positive for Pros in contrast to controls (Fig.7a and b,) supporting the observations reported in Fig.5 (whole brain images) in which we observed precocious neuronal differentiation in the absence of CLAMP. Moreover, in the null preparations we see fewer cells expressing Dachshund (Dac) which regulates OL development (Figs.7c and d) and an overall decrease in cell size compared to controls.

In summary, these results suggest that lack of CLAMP affects neurogenesis at multiple levels, from impacting mitotic chromosomal structure to affecting the neural stem cell niche homeostasis which leads to precocious differentiation of neural stem cells.

Discussion

The understanding of the molecular and transcriptional mechanisms by which neural stem cells function to ultimately build the brain is critical both to understand how brains develop. Signaling relays originating from the stem niche play an instructive role in the neurogenetic process and highlight the fact that the environmental context acts as an additional layer in addition to intrinsic developmental signals^{2,3}. In this study, we have defined a new role for the essential transcription factor, CLAMP, in nervous system development and NSC-niche interactions. Specifically, we show that CLAMP is important both for neuronal and glial cell generation and regulates NSC function *via* cell-intrinsic and NSC niche-dependent mechanisms that implicate niche glia.

Neural development and NSC function are intricately linked to niche glia and depend on support and nutrition from surface glia *via* Gbb-Dlp signaling, which sustains NSC proliferation and survival but also ensures the survival of surface glia⁵. Thus, there is an interdependency between NSCs and niche glia, ensuring overall homeostasis in the stem cell niche. In *clamp* null and *repo>clamp* RNAi larvae brains, surface glia numbers are reduced to the same extent indicating that lack of glial CLAMP affects gliogenesis (Fig.6d). Furthermore, the glial master gene, *gcm*, contains CLAMP binding motifs, indicating that CLAMP is likely to directly regulate gliogenesis (Fig.4e).

In the post-embryonic nervous system, *gcm* and *gcm2* are expressed in OL NSCs destined both for glia and neuron generation which they regulate *via* downstream mechanisms involving Dachshund (Dac)⁷⁹, a gene whose expression is reduced in the *clamp* null (Table 2 and Fig.7d). Moreover, both *gcm* and *gcm2* transcript levels were greatly reduced in the *clamp* null L3 brain and *gcm* contains CLAMP binding sites (Fig.4e). The OL contains a special population of NSCs which generate both glia and neurons (Lawfs), from a common progenitor population under the control of Notch signaling⁸⁰. Moreover, Notch is a key regulator which maintains the balance between symmetric/self-renewing and asymmetric NSCs divisions; loss of Notch function promotes precocious differentiation and premature neurogenesis⁵⁹. In the absence of CLAMP, *Notch* is at least two-fold downregulated both in the embryonic and L3 stages (Supplementary Table 1a and Table 2), and we determined that Notch has a nearby CLAMP binding site (Fig.4e). Overall, our data support a model in which CLAMP directs *Notch* and *gcm* signaling to regulate NSC proliferation and differentiation.

The formation of the OL was profoundly impaired in the *clamp* null, a phenotype accompanied by reduction of EdU⁺ NSCs (Figs.5a-d). We also observed increased numbers of Pros⁺ cells in the null OL (Figs. 5b and d, outlined area) and throughout the brain, consistent with lack of CLAMP promoting a neurogenic over a gliogenic fate. While glial *clamp* KD results in a partial reduction in the number of proliferating NSCs (EdU⁺, Fig.6e), it does not affect the number of Dpn⁺ NSCs/neuroblasts. These results suggest an effect on

the Dpn⁻ progeny of the NSCs such as the ganglion mother cells (GMCs). Also, it is possible that there are changes in the cell cycle kinetics that cause fewer NSCs to be actively proliferating. This possibility is consistent with the GO analysis which identified genes involved in cell cycle kinetics/transitions (Fig4.c). Moreover, CLAMP may have a regional role in OL neurogenesis because *tll* is a candidate direct target for CLAMP (Fig.4) and other transcription factors critical for OL neurogenesis such as *eya*, *dac* and *optix* are downregulated (see Table 2).

Under conditions of hypoxia and oxidative stress, niche glia form lipid droplets in order to reduce reactive oxygen species and inhibit oxidation of polyunsaturated fatty acids that will damage both the NSCs and glia⁶. In the *Drosophila* larval brain lipid droplets are formed exclusively by glial cells and not neurons, and specifically by subperineural and superficial cortex glial cells, with the latter most enriched²¹. Cortex glia are a very unique glial population. At the initiation of postembryonic neurogenesis, cortex glia ensheath each dividing neuroblast and their membrane acquires the appropriate morphology to accommodate the proliferating neuroblast and its progeny. Thus, these cells provide spatial organization for each NSC progeny cell, while at the same time nurturing and protecting NSCs^{5,21}. In the absence of CLAMP, there is a marked increase in the levels of brain LDs (Figs.6j-p) concomitant with the changes in NSC and surface glia numbers.

Thus, loss of CLAMP could be triggering a compensatory mechanism in glia through increase of lipid formation and/or abnormal lipid storage in glia based on our transcriptomics which showed enrichment for genes and GO categories implicated in insulin signaling, lipid mobilization and metabolism (Table 3 and Fig.4). Moreover, *wunen* (*wun*) a membrane-localized lipid phosphate phosphatase which regulates the levels of bioactive lipids and is expressed in SPG^{81,82} is a putative direct target of CLAMP (Fig.4e) which is upregulated in *clamp* nulls (Table 3). Thus, it is also possible that lack of CLAMP results in abnormal accumulation of LDs in surface glia through *wun* regulation. In the *clamp* null L3 brain there is also a dramatic upregulation in the levels of the glial-derived *dilp6*. dIlps- insulin-like peptides are released by

blood–brain barrier glial cells and act *via* the insulin/insulin-like growth factor pathway. Specifically *dIlp6* activates the PI3K/Akt pathway in NSCs to promote their reactivation^{8,60}. We noted an enrichment of differentially expressed genes which were part of the insulin signaling pathway (Fig.4c) such as *dIlp6* and upregulation and reduction in transcription of genes regulating cell cycle progression. Therefore, that lack of CLAMP results in impairment of NSC progression through the cell cycle/exit from quiescence, and thus stimulates a compensatory response from the NSC niche to overcome it.

Interestingly, we observed that large LDs are more often found around dividing neuroblasts in the *clamp* null brains (Figs.6l and m; Supplementary Fig.4). Also, NSCs lacking CLAMP show abnormal chromosomal behavior in larval mitotic chromosome preparations (Fig.7). During neuroblast divisions, the axis of polarity is regulated by intrinsic and external cues from the daughter GMC which induces polarization of the dividing NSC in order to orient its axis of polarity and its division plane⁸³. Loss of the proper orientation reduces the contact surface area with cortex glia and thus renders the proliferating NSC more sensitive to stress. Lack of CLAMP results in downregulation of the mitotic polarization determinants including *baz*, which is putatively under CLAMPs transcriptional control (Fig.4e). Therefore, the axis of polarity may be disrupted in *clamp* nulls which reduces the area of contact between cortex glia ensheathing the NSC. In budding yeast, the storage of excess lipids in LDs during anaphase is critical for proper cytokinesis and cell division and disruption of this process leads to defects in cell separation⁸⁴. Furthermore, accumulation of LDs was shown to be important for the progression of the cell cycle and proliferation in human cancer cells⁸⁵. Therefore, it is possible that the formation of large LDs around dividing neuroblasts in the *clamp* null is yet another compensatory mechanism that niche glia uses to ensure proper division of NSC.

We also observed that the *foxO* gene (Forkhead box, class O) is part of many of the cell cycle sub-categories that we identified from GO analysis, including the *insulin signaling category*, which is a known target of FOXO⁷⁴. FOXO belongs to a family of highly dynamic and conserved transcription factors and regulates

gene expression during processes such as apoptosis, cell-cycle transitions including negative regulation of G1/S and G2/M⁷⁵, both of which are highly enriched in our GO analysis. Overexpression of FOXO during larval development results in reduction both in cell size and number in the developing eye⁷⁴. In addition, it was shown that FOXO affects the temporal sequence and cell fate specification in a subset of olfactory neurons in *Drosophila*⁷⁶. Interestingly, in the *clamp* null embryo the *foxO* transcript is upregulated (L2FC: 0.46846, padj:0.000283, Supplementary Table 1a). Moreover, glia-derived DILP6 activates the Insulin Receptor (InR) on cholinergic neurons and induces Jeb activation which acts downstream to regulate FoxO activity in Insulin Producing Cells (IPCs)^{68,69}. Therefore, it is possible that some of the phenotypic effects that we observed in the *clamp* null mutant are related to dysregulation of *foxO* gene expression^{74,76}.

Glial cells can arise from different mechanisms, either from dedicated glioblasts or from NBs that have both glial and neuronal potential⁸⁶⁻⁸⁹. Our results indicate that CLAMP is important both for maintaining the proliferation of NSCs by acting in a cell-type specific manner (i.e, glia vs. NSCs) and in a cell-intrinsic mechanism that implicates regulation of chromosomal behavior and fate determinants such as *gcm* and *Notch*. In addition, CLAMP acts *via* niche-dependent mechanisms that implicate surface glia, Dilp6/insulin signaling and lipid droplet formation. Overall, here we have shown that CLAMP is an important regulator of NSC differentiation and of the stem cell niche.

Materials and Methods

Fly Strains

Fly stocks used in this study were as follows: *y¹w¹* (maintained in the Larschan lab); *clamp* null and *clamp* rescue lines, both of which have been generated by Urban et al.(2017) and described therein²⁵. Briefly, the *clamp* null, is a CRISPR-Cas9 generated frameshift mutation which abolishes CLAMP protein expression, whereas, the *clamp* rescue was generated by introduction of a full *clamp* rescue construct on chromosome 3L. For the RNAi experiments the following lines were used: *repo-GAL4*, *elav^{c155}* (*elav-GAL4*) and UAS-*clamp-RNAi*, all obtained from the Bloomington Stock Center. All crosses were done at 25°C and a 12-hour light/dark cycle.

Immunostaining

The following antibodies were used: Rabbit anti-CLAMP (1:200, Abcam custom made), mouse anti-Repo (1:50, Developmental Studies Hybridoma Bank (DSHB) 8D12), rabbit anti-DCP1 (1:100, Cell Signaling), rat anti-Deadpan (Dpn, 1:100, Abcam), rat anti-Elav (1:50, DSHB), mouse anti-Prospero (Pros, 1:30, DSHB), mouse anti-Dachshund (Dac, 1:40, DSHB), PCNA (PC10, Abcam), Nc82 (1:10, DSHB). Secondary antibodies used were Alexa Fluor Conjugated A405, A488, A568, A647 (1:1000, Invitrogen). A standard staining protocol was used.

Quantification of glia

To quantify superficial glia, 3-μm projections (z-projections of three 1-μm optical sections) of the dorsal surface of third instar larval brain hemispheres stained for Repo were used and quantified manually using Adobe Photoshop. Six hemispheres were quantified for each genotype.

Image acquisition and processing

Fluorescent images were acquired using an inverted Olympus FLUOVIEW FV1000 confocal laser scanning microscope. Adobe Photoshop was used to adjust brightness and contrast in images and ImageJ (or Fiji (Schindelin et al., 2012)) was used to analyzed images while Adobe Illustrator was used to compile figures.

EdU Incorporation

EdU incorporation in proliferative brain cells, was performed using Click-It Edu Alexa Fluor A488 (Thermo Fisher Scientific, MA, USA) in an adaptation of Tastan and Liu (2015)⁹⁰ method. Briefly, samples were dissected in ice-cold Graces media and then transferred in Graces media containing 5µg/ml of EdU, for 2 hours at room temperature. Then, samples were washed in 0.1% PBST and fixed in 4% PFA in 0.1% PBST, for 20 min at room temperature. Following, tissues were washed in 5% HINGS in 0.1 % PBST and then permealized in 0.5% PBST for 20 min (RT). After this step, the EdU Click It steps were followed according to the manufacturer's instructions and then additional antibody staining was followed as described above.

Brain Lipid Staining

Neutral brain lipids were stained using the LipidTOX dye as described in Bailey et al. (2015)⁶. Briefly, tissue was dissected in ice-cold PBS, and fixed overnight in 2% PFA in phosphate buffer with lysine at room temperature. Following PBS washes, the samples were permealized in 0.2% PBST, and stained with DAPI. Then followed LipidTOX Red (1:1000) staining with overnight incubation. Following PBS washes, samples were mounted in ProLong Gold for imaging.

Larval Staging

Third Instar larvae crawling on the vial walls were selected to be mid/late stage using the bromophenol blue method adjusted from Maroni and Stamey (1983)⁹¹, using 0.05% w/v bromophenol blue-containing fly food. To control for variations in brain size due to crowding, experiments were done under controlled density conditions. Specifically, a set number of mated males and females were allowed to mate for 5 hours and then removed from the vial. Additionally, the progeny number was evaluated to validate that we did not have large deviations between control and *clamp* mutant larvae numbers.

RNA extraction and RNA sequencing

Mid/late third instar larvae were staged using the bromophenol blue method and larval brains were dissected in ice-cold PBS, collected in TRIzol until RNA extraction was performed using the phenol-chloroform method. Each biological replicate was generated by pooling at least 12 larval brains. mRNA-focused sequencing libraries were constructed from total RNA using the Illumina Truseq RNA Library kit v2 according to the manufacturer's instructions (no strand-information). Paired-end 100 bp or 50 bp was performed on an Illumina HiSeq2500 sequencer in High-Output mode at the MGH NextGen Sequencing Core. Four biological samples were used per experimental condition.

RNA sequencing data analysis and Differential Gene Expression Analysis

RNA-seq reads were processed using FastQC⁹² with default parameters to check the quality of raw sequence data and filter out sequences flagged for poor quality, and aligned using HISAT2⁹³. Then read counts per gene were generated via HTSeq⁹⁴ using the *Drosophila melanogaster* genome dm6 (BDGP6) genome as reference. Differential gene expression analysis was performed using DESeq2⁹⁵. *gProfiler* was used for GO analysis⁹⁶. Using TIMEOR⁹⁷ the temporally differentially expressed genes were displayed in interactive

expression clustermaps along the developmental time scale (see Supplementary files: *Embryo_heatmap* and *L3_brain_heatmap*), from which additional, GO analysis was performed by TIMEOR *via* clusterProfiler⁹⁸ to evaluate for possible gene networks.

Clamp Binding motifs

We used FIMO⁴³ to identify individual CLAMP motif occurrences within ± 1 KB from the transcription start and end sites for all genes within each gene trajectory cluster. The position weight matrix used⁴⁴ highlights the CLAMP motif independently of GAF binding potential. The specific command used is: (*fimo --oc \$OUTPUT_DIR/CLAMP_PWM.txt \$geneList*)

Western Blotting

Larval brains were dissected from wandering third instar larvae in ice-cold 0.7% saline and transferred in PBS + 0.3% Triton X-100 on ice. Liquid was carefully removed, then samples were frozen in liquid nitrogen and stored at -80°C until use. Approximately ten brains were collected per biological replicate. For protein extraction, 50 μ L of ice-cold RIPA buffer (150 μ L 1M Tris-HCl pH 8, 12 μ L 0.5M EDTA, 0.003 g sodium deoxycholate, 15 μ L 20% SDS, 90 μ L 5M NaCl, 30 μ L 50x protease inhibitor, and 2700 μ L water) was added to each sample. Samples were homogenized using an electric handheld homogenizer and disposable plastic pestle. After homogenization, the pestle was rinsed with an additional 50 μ L of RIPA for a total volume of 100 μ L per tube. Samples were rotated at 4°C for 1-2 hours. Protein concentration was quantified using the Qubit Protein Assay kit (Thermo Fisher Scientific).

Statistical Analysis

Excel and GraphPad Prism were used for statistical analysis. Bar graphs were generated using the mean and standard error of the mean (SEM) for each sample. Statistical significance was determined using an unpaired Student's t-test.

Materials & Correspondence

Data and materials generated in this study are available upon request from the corresponding authors. Transcriptomic data have been deposited in Gene Expression Omnibus (GEO) Database, under the accession number GSE148704.

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958 **Acknowledgements**

959 We thank Dr. Edward A. Kravitz for providing MAT laboratory space and contributing to supply costs for
960 completion of this study, Dr. Yick-Bun Chan for experimental support and assistance with DEG analysis
961 and Dr. Yiannis Savva for experimental support. We would also like to thank Dr. Séverine Trannoy for
962 critical reading of this manuscript. We thank the Harvard Medical School Neurobiology Department and
963 the Neurobiology Imaging Facility for consultation and instrument availability that supported this work.
964 This facility is supported in part by the Neural Imaging Center as part of an NINDS P30 Core Center grant
965 #NS072030. This work was supported in part by a Harvard Medical School Fix Fund Postdoctoral
966 Fellowship to M.A.T and by US National Institute of Health grants to E.N.L and E.A.K (R35 GM126994-
967 01 and R35 GM118137).

969 **Contributions**

970 MAT and E.N.L conceived the study and M.A.T designed the study. M.A.T performed
971 immunohistochemistry and imaging, collection of RNA-seq samples and analysis of RNA-seq DEG results.
972 L.X contributed to sample collection for RNA-seq experiments and immunohistochemistry, E.N
973 contributed in size experiments and A.M.C contributed in RNA-seq data analysis. M.A.T prepared figures
974 and wrote the original draft while M.A.T and E.N.L curated the manuscript with input from all authors.

976 **Declaration of Interests**

977 The authors declare no competing interests.

Figure 1 CLAMP is ubiquitously expressed throughout the L3 nervous system and is present in proliferating neuroblasts. (a) Temporal expression data from modEncode (Flybase) reveal that *clamp* expression levels decrease as embryonic development proceeds and as the maternal transcripts are used-up and reach a nadir at 18-20 hours after egg lay, which is then followed by a sharp increase just before embryonic development is completed. During the larval stages *clamp* expression decreases until L3, where it slowly starts to increase again before the pupa stage. (b) Tissue specific L3 expression data, reveal that *clamp* is most highly expressed in the developing imaginal disks, the CNS and also the fat body. (c-g) CLAMP is expressed both in the L3 central nervous system, specifically in the optic lobe (OL, yellow dashed outline), central brain, (CB) and in the ventral nerve cord (VNC). (c, d) CLAMP is present in the nucleus throughout the nervous system as seen by co-staining with DAPI and Nc82, which marks the synaptic processes. In the VNC it is also observed in the neuronal processes (c, arrows). Panels e-g show individual staining channels. (h-n) Higher resolution images reveal high expression of CLAMP in the developing CB and OL and specifically in proliferating NSCs marked by Deadpan (Dpn) and Proliferating cell nuclear antigen (PCNA). (j) Magnification of the boxed area in panel i, reveals that CLAMP is present in cycling NSCs during different cell cycle phases according to the PCNA nuclear distribution. Star (*) in panels k and m indicates an artifact. Scale bars: 50 μ m and 25 μ m in j.

Figure 2 Lack of CLAMP results in growth deficits both in the body and the nervous system (a, b) Mutant *clamp* null L3 larvae are considerably smaller in size, compared to control L3s (Mean length $\pm$ SEM; Control: 3.369 ± 0.06031 , $n=14$; *clamp* null: 3.041 ± 0.04371 , $n=20$; t test $p < 0.0001$). (c, d) This reduction is maintained at the CNS level of *clamp* null L3 larvae (Mean normalized brain area $\pm$ SEM; Control: 1.001 ± 0.0485 , $n=20$; *clamp* null: 0.601 ± 0.04052 , $n=20$; t test $p < 0.0001$). (e) Western blot for CLAMP in samples from L3 brain lysates from control and *clamp* null L3 showing the absence of CLAMP protein ($n=2$ per condition). Scale bars: 1 mm in a; 50 μ m in c.

Figure 3 Lack of CLAMP greatly affects brain development and causes defects in optic lobe formation. (a, b and e, f) Brain development in the *clamp* null L3 is severely impaired from the early L3 stage, while even at the mid/late L3 stage, *clamp* null brains resemble early L3 control brains. (c, d and g,h) Full z-stacks of L3 brains showing the distribution of Deadpan (Dpn) -positive NSCs areas, reveals that NSCs development both in the central brain (CB) and the optic lobe (OL) of the null are impaired, starting from the early L3 stage and continuing to the mid/late L3 stage. Especially impaired is the OL development as seen by the reduction in the Dpn+ area indicated with the white dashed outlined areas. EAD: eye antenna disk. Panels a, b, e and f are 3 optical sections whereas c, d, g and h are full z-stacks throughout the brain. Scale bars: 50 μm.

Figure 4 CLAMP is implicated in transcriptomic networks that regulate the proliferation and differentiation of NSCs. (a) Experimental workflow for RNA sequencing data generation and DEG meta-analysis. Embryo panel is scanning electron image of a late stage embryo from⁹⁹. (b) Venn diagram of DEGs in the *clamp* null embryo and L3. (c, d) GO analysis of the *clamp* null DEGs revealed high

1024 enrichment in categories related to cell cycle dynamics and neural development. (e) Find Individual Motif
 1025 Occurrences (FIMO) analysis of the DEGs for the CLAMP binding motif, revealed that critical regulators
 1026 of neural development and mitosis are putative targets for CLAMP binding both at the embryonic and L3
 1027 stage.

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Figure 5 Lack of CLAMP impairs neural stem cell proliferation and promotes precocious differentiation. (a-d) Proliferation of NSCs as seen by EdU labeling, is highly reduced in the *clamp* null brain, with the most evident impact being on OL NSCs (panels a and c, dashed lines indicate the OL). In parallel, nuclear Pros⁺ clusters indicate acquisition of neuronal fate, are more evident in the *clamp* null brains (panels b and d, arrows). (e) Both the total number of proliferating stem cells (EdU⁺ Mean $\pm$ SEM, Control: 323.6 ± 4.679 ; *clamp* null: 50.40 ± 3.174 ; $p < 0.0001$; $n = 10$) and of proliferating Dpn⁺ NSCs is reduced (Dpn⁺EdU⁺ Mean $\pm$ SEM; Control 102.6 ± 2.141 ; *clamp* null 11.50 ± 0.3727 ; $n = 10$). (f) The reduction in total Dpn⁺ cells (Dpn⁺ Mean $\pm$ SEM, Control: 191.9 ± 5.313 ; *clamp* null: 57.90 ± 1.402 ; $p < 0.0001$; $n = 10$), is accompanied by a respective increase in Pros⁺ cells in the *clamp* null brains (Pros⁺ Mean $\pm$ SEM, Control: 47.1 ± 1.716 ; *clamp* null: 148.3 ± 2.629 ; $p < 0.0001$; $n = 10$). (g, h) Additionally, despite having an increase in the neuronal marker Elav in the *clamp* null brains, the OL development is impaired compared to controls (outlines). (i, j) Apoptotic cells marked with DCP1 are more in the OL of the null compared to a more ubiquitous distribution in the control. CB, central brain; OL, optic lobe; VNC: ventral nerve cord. Scale bars: a-d, 100 μ m; g and h, 50 μ m.

Figure 6 Glial-CLAMP surface glia generation and NSC proliferation (a-e) Surface glia (SG) is reduced by ~25% in the *clamp* null brain compared to controls (% SG Repo⁺ ± SEM, *Control*: 99.98 ± 5.328; *clamp* null: 75.37 ± 1.843; n=6) whereas, (e-i) proliferating NSCs (EdU⁺ ± SEM, *Control*: 323.6 ± 4.679; *clamp* null: 50.40 ± 3.174) and NSCs (Dpn⁺ ± SEM, *Control*: 191.9 ± 5.313; *clamp* null: 57.90 ± 1.402) are also reduced in numbers. Glial-specific *clamp* knock-down (*repo>clamp RNAi*) promotes a similar reduction in SG numbers (% SG Repo⁺ ± SEM, *repo>clamp RNAi* 131.0 ± 3.162) and promotes a reduction in proliferating NSCs (*repo>clamp RNAi* EdU⁺ ± SEM: 277 ± 6.572; Dpn⁺ ± SEM: 181 ± 3.964), but to a lesser extend as the one seen in the *clamp* null. Neuronal-specific *clamp* KD (*elav>clamp RNAi*) does not affect NSC proliferation and numbers NSCs (*elav>clamp RNAi* EdU⁺ ± SEM: 316. ± 9.519; Dpn⁺ ± SEM: 217 ± 14.45),. (j-p) Staining with the neutral lipid-specific dye LipidTOX, revealed an altered distribution of LDs in the *clamp* null brain compared to controls and (p) a ~50% increase in LDs in the *clamp* null brain, compared to controls (% LipidTOX signal ± SEM; *Control*: 100 ± 11.86; *clamp* null: 149.8 ± 8.599, n=6). (n-o) This increase is also evident in the panels showing a full z-stack for each condition. Panels l and m, represent magnification of the boxed areas in panels j and k respectively, where, large LD formations are observed surrounding dividing NSCs in the *clamp* null. Scale bars 100 µm.

Figure 7 Lack of CLAMP results in aberrant mitotic chromosome morphology (a,b) Chromosomal preparations of mitotic neuroblasts from L3 brains reveal the presence of mitotic chromosomes with altered morphology in the *clamp* null (rectangle in b). Additionally, in the *clamp* null condition more Pros⁺ positive nuclei are present, signaling more neuronal differentiation compared to controls. (d, c) Higher magnification images showing that mitotic NSC chromosomes in the *clamp* null in their majority are hyper-condensed and stubby (c, yellow rectangles) while the nuclei appear smaller compared to controls. Additionally, in the *clamp* null preparations, less nuclei are positive for Dac, a regulator of OL development. Scale bars, 100 μm.

Gene Name	log2FC	padj
<i>elav</i>	0.44	0.043
<i>ewg</i>	NA	NA
<i>clamp</i>	-1	6.39E-14
<i>CG12299</i>	-0.70	0.0001
<i>gro</i>	-0.88	2.45E-06
<i>CG8924</i>	-1.12	1.15E-10
<i>JIL-1</i>	NA	NA
<i>osk</i>	-4.76	1.67E-11
<i>ac3</i>	0.54	4.87E-05
<i>cdc16</i>	-0.83	0.005
<i>Ibfl</i>	-0.78	0.0002
<i>Bcl7-like</i>	-0.93	2.92E-11

Table 1: Functional interactors of *clamp* as identified by Haussman et al., 2008

<i>Gene ID</i>	<i>Gene Name</i>	<i>Log2FC</i>	<i>padj</i>	<i>Function</i>
FBgn0044047	<i>Dilp6</i>	-3.028	4.38E-42	OL development/Glia Neuroblast interactions/Insulin Signaling
FBgn0003720	<i>tlf</i>	-1.71	1.89E-31	OL development
FBgn0031745	<i>rau</i>	-1.373	3.42E-18	Glial cell development/differentiation of neurons and glia in the developing eye.
FBgn0014179	<i>gcm</i>	-1.281	5.90E-12	Glial cell development-promotes gliogenesis over neurogenesis
FBgn0010433	<i>ato</i>	-1.246	3.88E-15	OL development
FBgn0000529	<i>bsh</i>	-1.155	1.85E-24	OL development
FBgn0019809	<i>gcm2</i>	-1.154	3.97E-20	Has a redundant role with the product of <i>gcm</i> in the differentiation of glial cells and subset of neurons ⁷⁹ .
FBgn0031375	<i>erm</i>	-1.116	2.76E-19	Cell fate regulator during neurogenesis.
FBgn0000163	<i>baz</i>	-1.105	6.44E-10	Bazooka forms a complex with par-6 and aPKC. Functions in cell polarization and ACD.
FBgn0021776	<i>mira</i>	-0.99	3.16E-07	Neuroblast proliferation.
FBgn0000137	<i>ase</i>	-0.972	1.54E-05	Type I NB attributes.
FBgn0263512	<i>Vsx2</i>	-0.956	9.10E-23	OL development.
FBgn0010382	<i>CycE</i>	-0.951	0.00131	Cell cycle dynamics.
FBgn0001983	<i>wor</i>	-0.94	3.01E-05	Worniu, neuroblast attributes.
FBgn0000320	<i>eya</i>	-0.909	2.05E-08	OL development /insulin producing cells differentiation.
FBgn0000404	<i>CycA</i>	-0.855	0.010084	Cell cycle progression including G2.
FBgn0025739	<i>pon</i>	-0.847	3.66E-07	Contributes to asymmetric localization of numb and the subsequent suppression of Notch signaling.
FBgn0002973	<i>numb</i>	-0.845	1.67E-15	Acts via Notch signaling, asymmetric division control.
FBgn0005655	<i>PCNA</i>	-0.824	0.00019	Cell cycle dynamics.
FBgn0011674	<i>insc</i>	-0.788	1.44E-06	Spindle orientation; asymmetric divisions.
FBgn0037913	<i>fabp</i>	-0.72	1.85E-06	Expressed in lipid droplet-forming glial cells.
FBgn0013469	<i>klu</i>	-0.713	0.00013	Klumpfuss (<i>klu</i>) is a temporal fate determination.
FBgn0005677	<i>dac</i>	-0.707	4.97E-11	OL development.
FBgn0010109	<i>dpn</i>	-0.665	0.00245	Neuroblast self-renewal -interacts with <i>klu</i> and <i>erm</i> .
FBgn0004647	<i>Notch</i>	-0.642	0.0018	OL development/Stem cell proliferation.
FBgn0000411	<i>Dichaete (D)</i>	-0.634	2.56E-08	Nervous system development and OL development.
FBgn0044324	<i>Chro</i>	-0.61	0.00021	Neuroblast proliferation. Functions downstream of InR/PI3K/TOR signaling and regulates expression of quiescence-inducing Pros to permit NSC reactivation.
FBgn0005427	<i>ewg</i>	-0.533	1.06E-06	Neurogenesis/Synaptogenesis.
FBgn0040080	<i>pins</i>	-0.52	0.008985	Partner of Inscutable; involved in ACD and establishment of cell polarity.

<i>Gene ID</i>	<i>Gene Name</i>	<i>Log2Fc</i>	<i>padj</i>	<i>Function</i>
FBgn0000014	abd-A	2.983	0.00019	Controls the end of neuroblast proliferation.
FBgn0025879	Timp	2.299	9.57E-23	Coordinates synaptogenesis
FBgn0001258	Ldh	2.353	2.43E-22	Lipid metabolism.
FBgn0003117	pnr	2.106	0.008812	Activator of <i>proneural</i> achaete-scute complex genes.
FBgn0011746	ana	1.831	3.97E-20	Encodes a secreted glycoprotein that is expressed in glia and at lower levels in neuroblasts, <u>suppresses neuroblast proliferation</u> .
FBgn0032006	Pvr	1.742	8.56E-22	Glia development /neuroblast proliferation.
FBgn0016032	lbm	1.576	3.84E-21	facilitates synapse assembly
FBgn0037989	ATP8B	1.664	2.18E-20	Flippase, translocates phospholipids from one side of a membrane to the other forming new membrane structures, especially vesicles.
FBgn0031461	daw	1.502	2.82E-05	Regulates insulin release from IPC neurons.
FBgn0041087	wun2	1.249	3.17E-20	Encodes a lipid phosphate phosphatase; expressed in astrocytes.
FBgn0086677	jeb	1.224	1.37E-06	Functions as signaling ligand for the product of <u>Alk</u> .
FBgn0066101	LpR1	0.899	3.91E-21	Cellular uptake of neutral lipids from the circulating protein encoded by apolpp.
FBgn0000416	Sap-r	0.889	2.23E-29	Lysosomal Lipid homeostasis.
FBgn0000546	EcR	0.847	6.72E-06	Ecdysone Receptor.
FBgn0040505	Alk	0.692	0.00014	Employs Ras/ERK and PI3K signalling pathways, functions in multiple contexts including synaptic signaling.
FBgn0001297	kay	0.699	7.62E-12	Specifically expressed in glia.
FBgn0039332	alrm	0.635	0.00038	Glial related.
FBgn0000370	crc	0.61	3.83E-17	Coactivator of the product EcR, promotes expression the molting peptide hormone encoded by ETH.
FBgn0016078	wun	0.558	2.85E-07	Lipid phosphate phosphatase.
FBgn0013342	nSyb	0.506	3.24E-05	Synaptic signaling.
FBgn0030574	sbm	0.504	0.002373	L-type amino acid transporter expressed in glia; essential for proper timing of development and brain growth ⁴¹ .

Table 3: UP-regulated genes in the clamp null L3 brain