The Role of Scarecrow in Eliminating Central Brain Neuroblasts of *Drosophila melanogaster*

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SUMMARY

During development, the behavior of stem cells is tightly regulated to give rise to specific cell types and tissues and ensure appropriate organismal size. In some cases, stem cell regulation can be disturbed, giving rise to tumors, growth defects, tissue malformation, or improper injury recovery. Understanding the molecular mechanisms underlying the control of stem cells is therefore critically important to preventing and treating these defects.

During central nervous system (CNS) development in the fruit fly *Drosophila melanogaster*, neural stem cells, called neuroblasts, divide asymmetrically to self-renew and produce a daughter cell that will eventually symmetrically divide into neurons or glia. The population of neuroblasts decreases as development approaches completion, and all neuroblasts are eliminated before eclosion[12,23,27]. Preliminary data from our lab suggests that a transcription factor, scarecrow (scro), may play a role in the termination of a subset of neuroblasts.

Our lab has shown that knockdown of scro in neuroblasts results in the persistence of neuroblasts in the adult fly brain. These neuroblasts persist past the normal time of their elimination and throughout adulthood. When scro is knocked down in either type I neuroblasts or in a specific subtype of neural progenitors called intermediate neural progenitors (INPs), ectopic neuroblasts persist into adulthood. Taken together these data lead to the hypothesis that scarecrow terminates neurogenesis in type I neuroblasts and INPs through the restriction of the cell fate of ganglion mother cells (GMCs).

BACKGROUND

Stem Cells

Stem cells are undifferentiated cells that have the capability of dividing to produce the differentiated cell types important for growth during development and the maintenance of tissue homeostasis in adulthood. The pluripotent nature of stem cells makes them an attractive option for treatment of a variety of degenerative diseases and injury. Stem cells are currently being explored as tools for treatment of spinal cord injury, heart disease, neurodegenerative diseases (i.e. Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Parkinson's Disease), as well as a number of other medical conditions[1,2,8]. By understanding stem cell behavior and its regulation, their use as a therapeutic treatment can be realized. However, an understanding of the basic biology of stem cells is important for informing clinical application.

During development, stem cells undergo a series of symmetric divisions, where each division produces two stem cells, allowing them to increase their population. Eventually, these stem cells switch to asymmetric divisions to give rise to a more differentiated daughter cell and also self-renew. As the multicellular organism develops, a majority of these stem cells must terminate proliferation in order to ensure proper size of tissues and cellular components of the organism (i.e. brain, blood cells). Specifically, during development of the nervous system, neurogenesis, neural stem cells (NSCs) asymmetrically divide to give rise to the major cells and components of the central and peripheral nervous system[9].

Drosophila Neuroblasts in Development

Neural stem cells in *Drosophila* are called neuroblasts (NBs) and provide a tractable genetic model for studying neural stem cell behavior and their cell lineages *in vivo*. A single central brain NB divides up to a hundred times and can be identified by its large size, stereotypic position, and unambiguous molecular markers[9]. These factors, coupled with the relatively few (200 NBs) in the central brain, makes them amenable to analysis over time. Moreover, individual NBs express combinations of intrinsic factors that determine their proliferation rate, the types of neurons and glia they produce, and when they are ultimately eliminated[11,18].

The different subtypes of NBs located in the central brain include type I, type II, and mushroom body NBs (MBNBs). These subsets are defined by their different patterns of proliferation (Figure 1). Type I and MBNBs divide to self-renew and produce one ganglion mother cell (GMC). The GMC then divides once to produce two neurons and/or glia. Type II NBs also divide asymmetrically to self-renew but rather than producing a GMC, they generate an intermediate neural progenitor (INP). This INP is a transient amplifying cell, which asymmetrically divides to self-

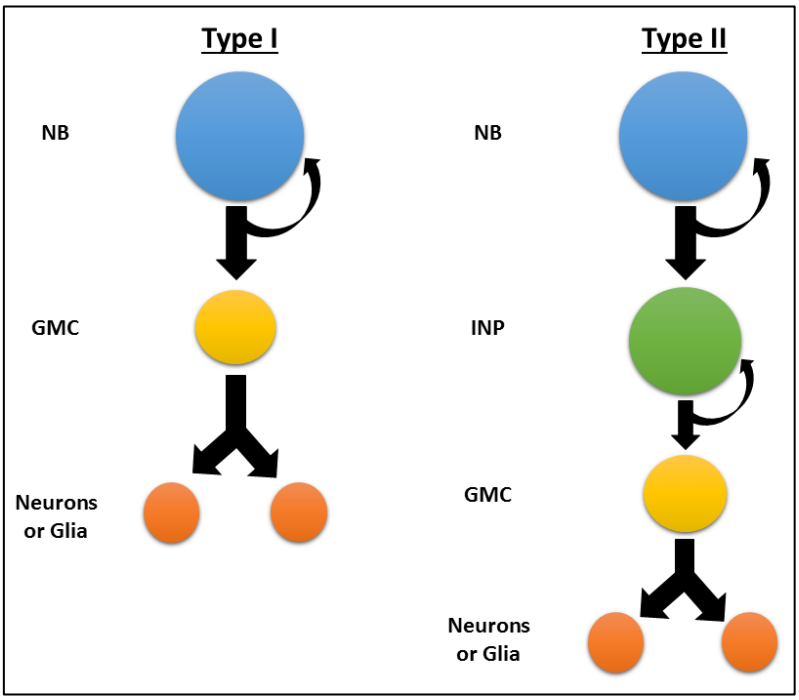

Figure 1: Type I and Type II NB Cell Lineage.

renew and also gives rise to a GMC. There are approximately 90 type I NBs and only eight type II NBs per lobe in the developing central brain, but due to their cell lineages, type II NB progeny account for a larger number of neurons than type I NBs[9]. The eight MBNBs (four per lobe) differ from type I and type II NBs in their pattern of proliferation over the course of development.

The proliferation of NB lineages during development has been well characterized. NBs are specified and begin proliferating during the mid-embryonic stage. At the end of embryogenesis, a majority of these NBs enter a period of quiescence during which they temporarily cease divisions[5,12]. The eight MBNBs are exceptions and continue to divide through this phase of development. Once the larva hatches, the non-MBNBs reactivate and begin to divide[5]. These NBs continuously produce neurons and glia throughout larval stages and into the pupal stage.

During divisions, the asymmetric localization of factors to the basal or apical membrane of the dividing NB must occur. Asymmetric partitioning of Brat and Numb into immature INPs ensures their maturation and also prevents their reversion back into NB-like cells[28]. Brat is asymmetrically localized to the basal membrane of a dividing INP by Miranda, where it is then inherited by the daughter GMC[16]. Brat is essential for the proper timing of Pros nuclear localization as well as its asymmetric localization to the GMC. When Miranda is mutated or its expression is knocked down, Brat is no longer asymmetrically localized to the GMC. When Brat is not properly localized or it is mutated, this can lead to the presence of ectopic NBs through dedifferentiation of GMCs[16]. Meanwhile, Pins anchors atypical protein kinase C (aPKC) to the apical membrane while lethal giant larvae (Lgl) prevents the localization of aPKC to the basal membrane[15]. aPKC promotes NB self-renewal and is therefore only inherited by the daughter NB after asymmetric division occurs.

During the early pupal phases, non-MBNBs terminate (15-30h after pupal formation (APF)) while the MBNBs continue to divide throughout the later pupal stages (72-96h APF)[12,23]. To cease its developmental program, the NB must undergo cell death, permanently exit the cell cycle, or enter a period of quiescence. Adult neurogenesis does not occur in *Drosophila* as indicated by the absence of NBs in the fly brain after eclosion from its pupal case[23]. In contrast, some mammalian neural stem cells do persist into adulthood in order to be involved in adult neurogenesis[4].

Termination of Neurogenesis in *Drosophila*

It is necessary to terminate growth once development is completed. In *Drosophila,* the population of NBs is eliminated through a combination of apoptosis and terminal differentiation. Multiple genes and pathways have been implicated in controlling these processes[10,18,23]. Prior to their elimination, both MBNBs and non-MBNBs undergo a reduction in size prior to their elimination, which requires changes in metabolic signaling pathways[10,23].

MBNBs are eliminated via apoptosis during the late stages of pupal development and this process is regulated by the pro-apoptotic genes *reaper, hid,* and *grim*[23]. However, disruption of proapoptotic genes in MBNBs only allows for their transient persistence into the adult brain. Long term persistence of MBNBs requires the inhibition of both apoptosis and autophagy[23]. The timing of MBNB removal also corresponds to an increase in the nuclear accumulation of the transcription factor foxo, indicating that both pro-apoptotic and insulin signaling pathways contribute to the timely elimination of these NBs. In addition to MBNBs, some non-MBNB lineages have also been reported to undergo programmed cell death[13].

The mechanisms that trigger elimination of other NBs are less clearly established. The earliest of these processes includes a temporal series of transcription factors that control NB fate. This series begins during

embryogenesis and consists of the transitions from Hunchback (Hb) to Kruppel (Kr) to Pdm to Castor (Cas) to Seven-up (Svp). This cascade controls the temporal identity of NB progeny in a cell-cycle dependent manner[11]. Svp has been shown to play a role in the timely elimination of type I NBs by disrupting the nuclear localization of Prospero (Pros), a transcription factor that controls cell cycle exit and inhibits expression of genes involved in NB self-renewal. However, the mechanism by which Svp controls Pros has yet to be characterized. This temporal series has also been shown to control timely apoptosis of abdominal type II NBs in the ventral nerve cord, though this mechanism has yet to be demonstrated in the central brain[18].

Proper regulation of Pros is important to control the proliferation and differentiation of INPs in the type II NB lineage. Pros expression is absent from type II NBs and localized to the cytoplasm in INPs. Misexpression of Pros leads to the premature termination of INPs and dramatically reduces the number of neurons and glia produced[3]. Conversely, loss of pros leads to defects in neuronal differentiation and an accumulation of INPs[28]. In INPs, nuclear localization of Pros is regulated by a transcription factor, Earmuff (Erm), which is specifically expressed in INPs[32]. Erm is required for the restriction of INP fate in the type II NB lineage, as loss of Erm function results in INPs reverting to "NB-like cells"[28].

Additionally, NBs may be eliminated through a mechanism involving ecdysone mediated shrinking. This shrinkage occurs during pupal development as NBs slowly begin to shrink as they near elimination[10]. At this point during development, NBs have divided to produce most of their progeny and are close to elimination. Ecdysone, together with Mediator, uses a cell-autonomous and metabolism-independent process to induce a transition to oxidative metabolism of glucose which induces shrinkage of the NBs. This shrinkage eventually leads to the disappearance of NBs within the first 30-40 hours of pupal development[10].

Scarecrow

Our lab performed an RNAi screen for cell intrinsic factors that are involved in the elimination of MBNBs (Pahl and Doyle, unpublished). This was accomplished by crossing a fly line expressing *worniuGAL4*, a driver which expresses GAL4 in most NBs, to a line containing a UAS RNAi construct for specific genes of interest (Figure 2). Expression of the RNAi is activated in NBs when GAL4 binds to the upstream activating sequence (UAS) present in the promoter region and activates RNAi transcription. From the 65 RNAi fly lines that were screened, 10 lines exhibited adult NB persistence and two had premature loss of NBs. One such hit was transcription factor scarecrow (scro) (*UAS scro RNAi)*, which led to the persistence of NBs in freshly eclosed adult (FEA) brains when knocked down (Pahl and Doyle, unpublished).

The role of scro in *Drosophila* development is largely uncharacterized. Scro was initially identified as a NK-2 homeodomain containing transcription factor with sequence similarity to *tinman (tin),* and was shown to be expressed in the embryonic and larval CNS[30]. NK-homeobox genes have been characterized and identified based on shared homology within their homeodomain sequence[14]. These genes are involved in tissue specification and play diverse roles during development.

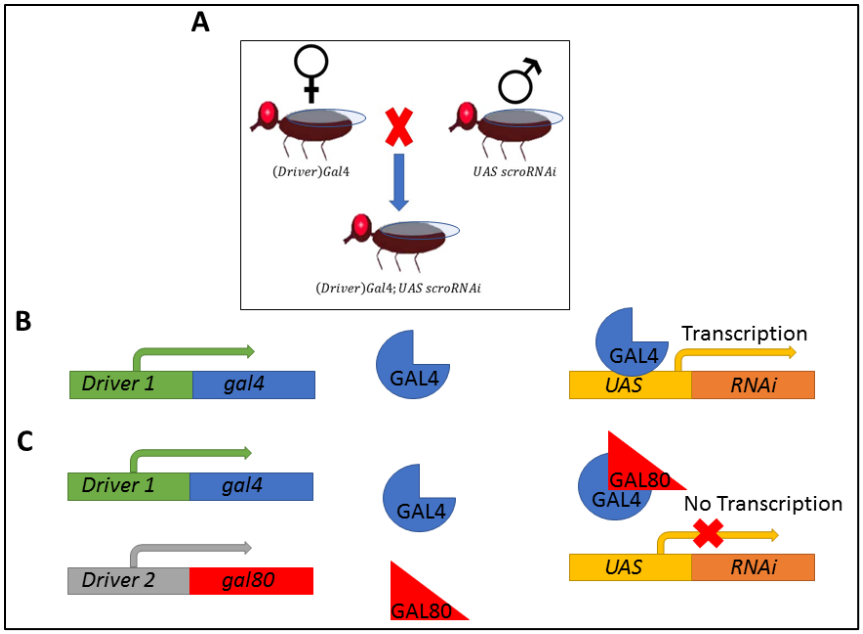

Figure 2: Gal4-UAS System. A) Example of *Gal4* and *UAS* cross and the resulting progeny B) How the Gal4/UAS system functions to promote transcription of a gene or RNAi. C) How the Gal4-Gal80/UAS system functions to conditionally repress transcription of a gene or RNAi.

Based on preliminary data obtained from the RNAi screen, I hypothesize that scro is expressed in the neural progenitors in the *Drosophila* central brain where it controls the termination of these neural progenitors during development. To investigate this hypothesis, I set out to characterize the expression of scro and elucidate its role during neurogenesis.

MATERIAL AND METHODS:

Fly Stocks

The following fly lines were used: *worniuGAL4 (*Chris Doe), *R9D11(erm)*GAL4[28], *asenseGAL4*[31] *(*gift from Cheng Yu Lee), and *worniuGAL4,asenseGAL80* [21], and UAS scro RNAi (Bloomington).
Virgin female *GAL4* containing flies were crossed with *UAS scro RNAi* male flies. Crosses were kept at 25 degrees in condos. Eggs from the crosses were laid on grape plates and plates were collected after 4-6 hours. L1 larvae were picked off the plates and transferred to vials. Larvae were kept at 25 degrees until the necessary developmental time point was reached (i.e. 72 hours after pupal formation (APF)).

Dissections and Immunohistochemistry

Fly brains were dissected and immediately fixed in 4% paraformaldehyde for 25-30 minutes. Brains were kept in blocking solution (10% Normal Goat Serum, 0.1% Triton in PBS) overnight. After washing, brains were then incubated in primary antibody for 48 hours at 4˚C, and then washed and incubated in secondary antibody for 48 hours at 4˚ C. Finally, brains were transferred to Diamond Anti-Fade (Thermofisher) until imaging.

The following primary antibodies were used at the indicated dilutions: chicken anti-GFP (1:1000) (Abcam), rat anti-deadpan (1:500) (Abcam), rabbit anti-scribble (1:1000) (gift of Chris Doe), mouse anti-prospero

(1:1000)(Developmental Studies Hybridoma Bank). The following secondary antibodies from ThermoFisher were used: goat anti-chicken Alexa Flour 488 (1:300) (Abcam), goat anti-mouse Alexa Flour 488 (1:300), goat anti-rat Alexa Flour 555 (1:300), and goat anti-rabbit Alexa Flour 633 (1:300).

Preparation of Specimens

Immunolabeled brains were mounted in Diamond Antifade (ThermoFisher) on a slide. To prepare a slide, two 22x22 #2 coverslips glued to either side. The sample was then placed dorsal side up in the center in 10 µL of Diamond Antifade and a 22x50 #1 coverslip was placed on top of the sample to create a bridge between the two smaller coverslips (Figure 3).

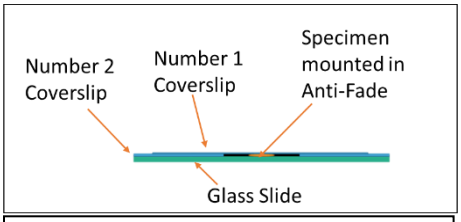

Figure 3: Sample Mounting

Imaging of Brains

Brains were imaged using a Leica SP8 laser scanning confocal microscope. A z-stack that encompassed each individual brain lobe was acquired. Set laser power intensities were used and detector gain was adjusted throughout the z-stack.

Images were analyzed using the ImageJ software package and statistical analysis was completed using Graphpad Prism.

RESULTS

Knockdown of Scro Leads to Long-Term Persistence of a Subset of Non-MBNBs

As mentioned, knockdown of scro with worniuGAL4 results in the presence of ectopic NBs in newly eclosed adults. I first examined 72 hours after pupal formation (APF), approximately 48 hours after non-MBNBs are normally eliminated, in scro knockdown brains (*scro* RNAi). As a wild type (WT) control, I crossed *worniuGAL4;pcnaGFP* virgin female flies with OregonR male flies. At this time point in control brains, only the MBNBs are present (Figure 4A). Actively dividing NBs were identified by the presence of the NB specific marker, deadpan, and expression of the transgene that marks proliferating cells, PCNAGFP. In scro RNAi 72h APF brains, the eight MBNBs (4 per lobe) were present as well as of 7.73±0.786 ectopic non-MBNBs (n=11)(Figure 4A, C). All of the ectopic NBs were located at the dorsal surface of the brain.

Next, I assayed brains expressing the *scro* RNAi in NBs from freshly eclosed adults (FEA). There were 8 ±2.16 persisting non-MBNBs present in the central brain (n=7) (Figure 4C). To determine whether these ectopic NBs persisted or were only transiently present, I assayed the number of ectopic NBs in control and scro RNAi brains at later time points. In 1 week old adult (WOA) scro RNAi brains, there were 3.17±2.66 persisting non-MBNBs in the central brain (n=12), substantially fewer than the FEA *scro* RNAi brains. Likewise, the 2 WOA brains showed 3.75±3.5 ectopic non-MBNBs (n=4). As expected, in the control brains, there were no NBs present in any of the brains from either FEA, 1 WOA, or 2 WOA. These persistent ectopic NBs were present only on the dorsal surface of the brain.

Multiple additional time points were assayed until the death of the fly at approximately 8 weeks. Ectopic NBs were found at all time points, though the number significantly decreased as the flies aged. Upon investigation of different structural components of the central brain, there appeared to be no aberrations and all structures were present. Based on these observations, I conclude that knockdown of scro expression alone allows for long-term persistence of NBs. Interestingly, I observed no obvious behavioral phenotype in scro RNAi flies; all scro RNAi flies could fly, crawl, and reproduce. Nevertheless, a more careful analysis may reveal changes in behavior.

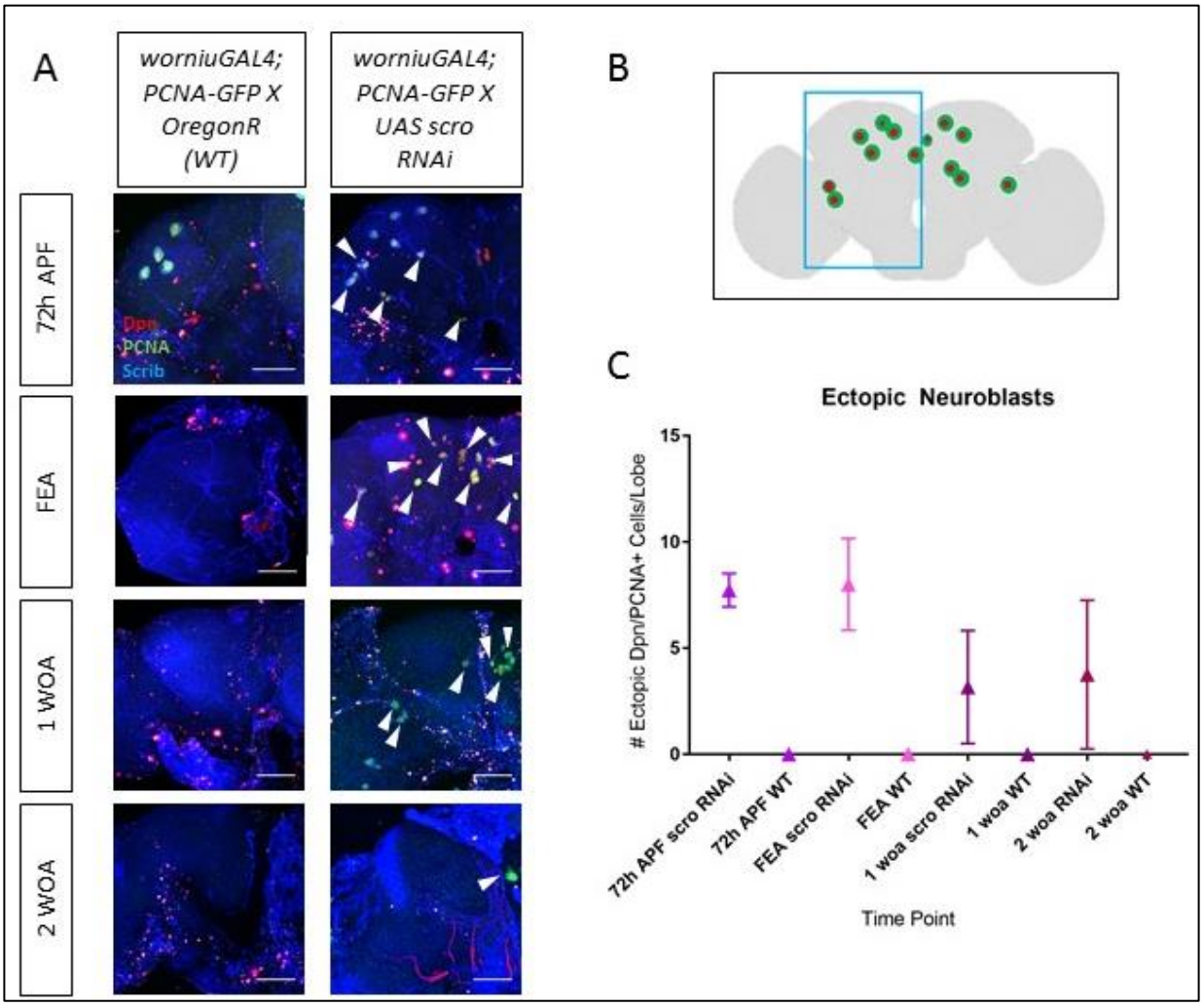

Figure 4: Knockdown of scro in NBs using worniuGAL4 and UASscroRNAi. A) All brains are labeled with deadpan (red), scribble (blue) and PCNA GFP (green). Ectopic NBs are marked with white arrowheads. B) Blue box denotes the region of the brain that was imaged. C) Quantification of ectopic neuroblasts after scro knockdown.

Knockdown of Scro in Subsets of Neural Progenitors Reveals the Identity of the Persisting Cells

NBs can be separated into several distinct classes based on the pattern of their division. I hypothesized that scro might affect a specific subset of NBs since knockdown of scro expression results in ectopic NBs stereotypically located at the dorsal surface of the brain. To determine the identity of the NBs that persist in scro RNAi brains, I knocked down scro in specific subsets of neural progenitors (i.e. type I NBs, type II NBs, and INPs) using three different lineage-specific GAL4 lines and assayed FEA brains for persisting NBs.

The specificity of these drivers was confirmed by crossing them to a *UAS mCD8 GFP* fluorescent reporter line. If driving scro RNAi in a specific population of neural progenitors leads to their persistence, then it is likely that scro is expressed in that subset and also plays a role in regulating their elimination.

I first used a type II NB specific driver, *worniuGAL4*, *aseGAL80*. Ase is expressed in all NBs except for the type II NBs. When Ase drives expression of GAL80, GAL4 activity is repressed in type I NBs, which leads to expression of the scro RNAi UAS construct solely in type II NBs. This driver was tested first because the ectopic NBs tended to be found in clusters. This would be expected of the type II NB lineages where both type II NBs and their mature INP progeny are Dpn positive[3]. However, when scro RNAi was expressed solely in type II NBs, we did not observe ectopic NBs (n=9). This result suggests that scro does not play a role in the elimination of type II NBs (Figure 5A).

Next, I drove scro RNAi with a driver specific to INPs (ErmGAL4). When scro was knocked down in INPs, this led to 1.43±0.98 ectopic Dpn positive cells in FEA brains on the dorsal surface of the brain (n=7). There were significantly fewer NBs present when compared to the knockdown of scro expression using the pan NBs worniuGAL4 driver. This finding indicates scro is acting in INPs, and that there is likely another subset of neural progenitors where it is also controlling termination of neurogenesis or the potency of the ErmGAL4 driver is weak.

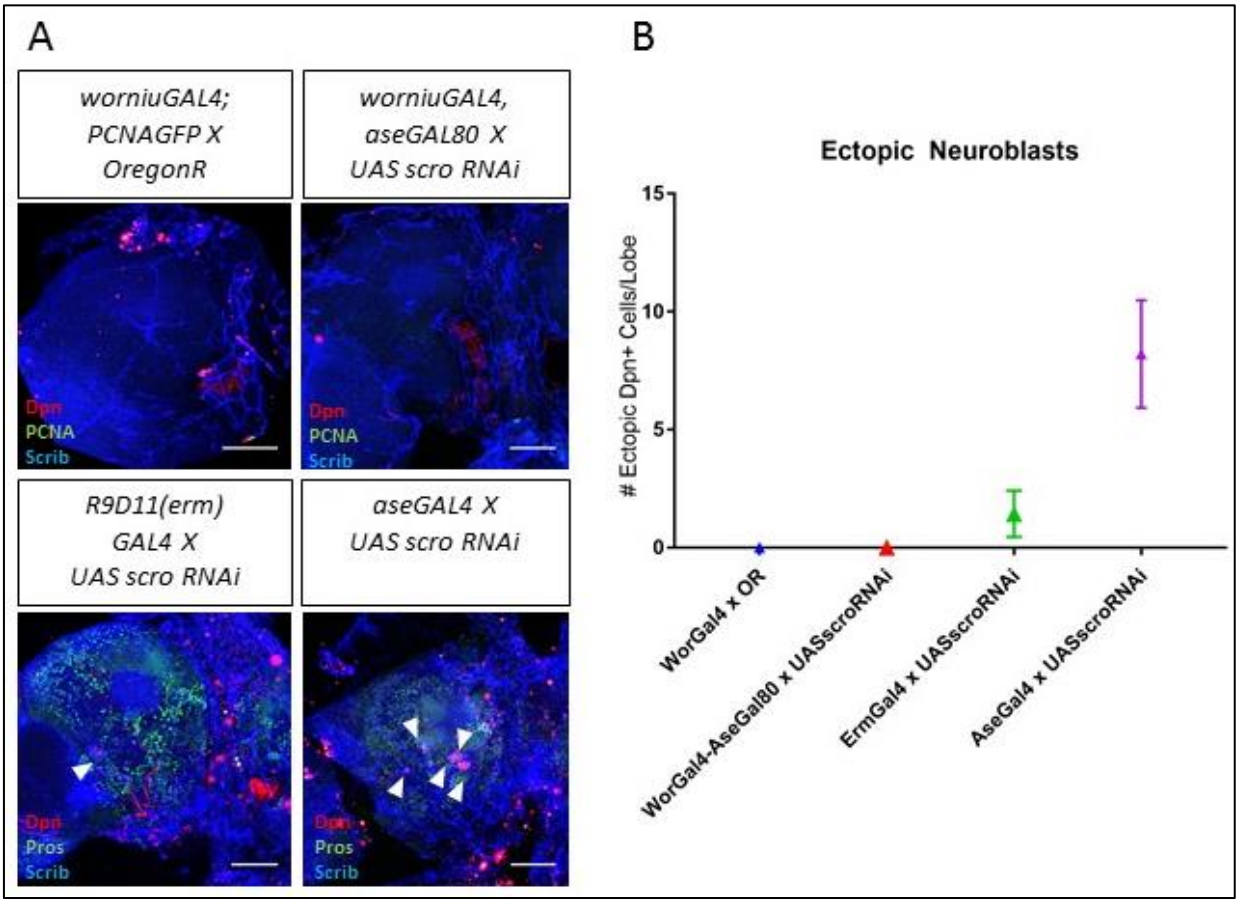

Figure 5: Knockdown of scro in specific neural progenitors. A) Ectopic NBs marked with white arrowheads. B) Quantification of ectopic neuroblasts after scro knockdown.

Finally, I drove expression of the scro RNAi with the *aseGAL4* driver, which is expressed in type I NBs, GMCs, and INPs. When scro is knocked down in these cells, 8.20±2.28 NBs persist in the FEA on the dorsal surface of the brain (n=5).

Taken together, these data demonstrate that scro plays a role in the timely elimination of neural progenitors by specifically restricting the developmental potential of small subset of INPs and type I NBs.

CONCLUSION AND FUTURE DIRECTIONS

Knockdown of scro in NBs results in the presence of ectopic NBs in the adult brain which persist late into adulthood and potentially never undergo termination. My findings suggest that scro is playing a role in eliminating a subset of INPs and type I NBs. One commonality shared by INPs and type I NBs is their self-renewal and production of a GMC during their asymmetric division. Scro may play a role in restricting the cell fate potential of GMCs and knockdown of scro may disrupt the expression of specific factors involved in this process. Future experiments and research should provide more detailed information on scro expression as well as on its role in NB elimination and cell fate restriction.

Exploration of Scro Related Proteins

Although the function of scro is poorly characterized, there are related homeodomain containing transcription factors (Nkx2.1 and Vnd) whose functions could suggest a role for scro.

Nkx2.1 is the mammalian ortholog of scro and is a member of the Nkx family of homeobox transcription factors[26]. Nkx2.1 has been shown to play a role in the early embryonic CNS development in vertebrates[17]. During development, Nkx2.1 null mutants exhibit defects in migration and axonal guidance and also lack a thyroid gland, pituitary gland, and ventral forebrain region, indicating that Nkx2.1 plays a role in the development of these specific tissues as well as neuronal migration[19,20]. Additionally, the mutant phenotype for Nkx2.1 indicates that Nkx2.1 acts as a tumor suppressor[29].

During neurogenesis in the mammalian cortex, NSCs are specified at the ventricular zone (VZ) and eventually their basal progenitor progeny and neurons will migrate to the subventricular zone (SVZ) or the cortical plate, respectively[4]. Nkx2.1 plays an important role in the specification of embryonic stem cells, especially within the region that gives rise to the sub-ventricular zone (SVZ) after endoderm formation. Nkx2.1 also controls the migration and specifies the identity of interneurons in the CNS[6,22,33]. More recently, it has been shown that Nkx2.1 has the ability to maintain the identity of embryonic neural stem cells in *in vitro* cell cultures[7].

Additionally, scro shows significant similarity to another *Drosophila* gene, ventral nervous system defective (Vnd), which is involved in dorsal ventral patterning of the CNS. Vnd is the most closely related protein to scro in *Drosophila*. Vnd is a transcription factor required for proper dorsal-ventral patterning in the embryo and is one of the earlier transcription factors that is expressed during embryogenesis[34]. Vnd also controls the specification of a subset of ventral NBs in the developing embryo, as Vnd mutants lack a number of neurons and exhibit defects in axonal patterning[24]. Further evidence suggests that Vnd has two isoforms that possess opposing functional properties: one isoform acts as a repressor and promotes NB identity while the other is an activator that promotes differentiation of neural progenitors[25]. This evidence suggests that it could also be possible that scro could possess opposing roles during different stages of development.

Though the information on these related proteins could suggest possible roles of scro during neurodevelopment, neither protein has been shown to play a role in the termination of neurogenesis and the elimination of neural stem cells. Therefore, further experiments will need to be done to confirm scro's function in the termination of neurogenesis.

Determine When and Where Scarecrow is Expressed During Development

Individual NBs adopt unique spatial and temporal identities. From my preliminary data, I suspect that scro is functioning in INPs and Type I NBs. Scro may be playing a role as a cell lineage restricted spatial or temporal factor. One critical aspect of scarecrow that needs to be clarified is its spatial and temporal expression pattern in the central brain during development. As mentioned, scro expression has been shown in the central brain during the larval stages of development[30], though its specific expression pattern throughout development remain unclear. Scro expression can be further characterized through the development of an antibody for scro, *in situ* hybridization, or through genetic manipulation of the genome (i.e. CRISPR) to create a fluorescently tagged form of Scro.

Determine the Role of Scarecrow During Development

Once a more detailed expression pattern of scro has been determined, the next step would be to examine the role scro plays within that subset of progenitors. The presence of persisting NBs in adult brains indicates that knocking down scro leads to disruption in the termination of neurogenesis.

In order to identify time points of interest, it will be important to test earlier developmental time points to identify when the number of NBs begins to vary from the wild type. This could be done using a temperature sensitive expression line expressing GAL4 and a temperature sensitive GAL80 allele, which blocks expression of RNAi at a permissive temperature but at a higher temperature, disrupts repression of the transcriptional activation function of GAL4 and allows for expression of the RNAi. By turning on this system at the specific points in development by shifting the flies to the non-permissive temperature, it is possible to determine when scarecrow expression is required for the proper elimination of NBs.

Another thing that is necessary to confirm the specificity of the scro RNAi is a scro null allele mutant. This mutant would confirm that the scro RNAi phenotype was due to knockdown of scro expression and not due to some non-specific knockdown of another gene. This mutant could be generated using CRISPR or imprecise excision of a P-element located in the scro locus.

Additionally, characterizing ectopic expression of scro by driving ectopic scro expression will determine if scro is sufficient to eliminate NBs prematurely. Based on my results, I would expect that over expression of scro would lead to a premature loss of NBs. The data from these experiments would aid in determining a possible role for scarecrow during the termination of neurogenesis in the specific cells where it is overexpressed.

Determine the Mechanism by Which Scro Regulates NB Termination

The final step in the characterization of scarecrow would be to fit scarecrow into the series of pathways that are involved in the termination of NBs. One possible way to determine this would be to test candidate pathways by looking at protein expression of pathway components following scro overexpression or knockdown. For example, due to the effects that scro knockdown has in INPs and Type I NBs, it can be hypothesized that scro is involved in the restriction of cell fate in GMCs or the asymmetric localization of proteins involved in cell fate restriction after NB division.

Scarecrow could be playing a role in the nuclear localization of Pros, playing a similar role to Erm, or disrupting asymmetric cell division. Scro could also act to prevent the dedifferentiation of neural progenitors and therefore restrict their cell fate. Determining the specific pathway that scro is acting and its interaction partners will further its characterization as a regulator of neurodevelopment in *Drosophila* and allow for exploration of related pathways in mammalian species.

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