Pipsqueak family genes dan/danr antagonize nuclear Pros and prevent premature decommission of neural stem cells in Drosophila third instar larval brains

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Abstract

Background

Neural stem cell fate is regulated for the systematic production of a precise quantity of neurons during the development of the central nervous system.

Results

Here we found that two *Drosophila* pipsqueak family genes, *distal antenna* (*dan*) and *distal antenna-related* (*danr*), promote the proliferation of neural stem cells, termed neuroblasts (NBs), in third instar larval brains. In the absence of Dan and Danr (Dan/Danr), the NBs produce fewer daughter cells with smaller MARCM clone (lineage) sizes. The larval brain NBs in *dan/danr* clones show premature accumulation of nuclear Prospero (Pros), which only appears in the *wildtype* (*wt*) terminating NBs at early pupal stage. The premature nuclear Pros leads to NBs cell cycle defects and NB marker loss. Removal of Pros from *dan/danr* MARCM clones prevents lineage size shrinkage and rescues the loss of NB marker expression. Our data show that the appearance of nuclear Pros is behind the downregulation of Dan/Danr in the *wt* terminating NBs. We demonstrate that Dan/Danr and nuclear Pros are mutually exclusive in NBs. In addition, Dan/Danr are partially required for the late temporal regulator, Grainyhead (Grh), in third instar larval brain NBs.

Conclusion

Together our study uncovers the novel function of Dan/Danr in NB cell fate maintenance. Dan/Danr antagonize nuclear Pros, preventing NBs from premature decommission in *Drosophila* third instar larval brains.

Background

The precisely regulated proliferative status of neural stem cells plays a pivotal role in neurogenesis. Any disturbances of neural stem cell fate will lead to progeny number defects (Holguera and Desplan 2018; Dray et al. 2021). *Drosophila* neural stem cells, termed neuroblasts (NBs), provide a unique model system to study the mechanism of how neural stem cells maintain their cell fates (Homem *et al.* 2014; Wu *et al.* 2019; Liu *et al.* 2020; Maurange 2020; Sang *et al.* 2022).

In central brains of *Drosophila* larvae there are two types of NBs: type I and type II (Bello et al. 2008; Boone and Doe 2008; Doe 2008; Izergina et al. 2009). Type I NBs represent the majority of neural stem cells (~ 90/lobe) and exhibit specific markers such as Asense (Ase) and Deadpan (Dpn). Type II NBs have a smaller population (8/lobe) and express Dpn, but not Ase (Boone and Doe 2008). All of these NBs

undergo proliferation at larval stages and terminate their respective stem cell fates at the early pupal stage (Maurange et al. 2008; Chai et al. 2013; Wu *et al.* 2019).

The cell fate of NBs needs to be precisely maintained along the developmental axis together with temporal regulation (patterning). Temporal regulation is originally defined as a cascade of transcription factors that are sequentially expressed in NBs and specify NBs temporal identities (Isshiki et al. 2001). Temporal regulators not only determine NB temporal identities but also control NB proliferative status (Maurange et al. 2008; Bakshi et al. 2020). Recently, many temporal regulation factors including the hormone receptor Ecdysone (Homem *et al.* 2014; Sood *et al.* 2021), signaling pathways such as Hh and Tor (Chai et al. 2013; Maierbrugger et al. 2020), and the post-transcriptional regulators Imp-Syn (Liu et al. 2015), have been detailed. These temporal regulation factors also act to maintain NBs proliferative status and are divided into two groups. The first group promotes and ensures the scheduled NB termination in the early pupal brain. This prevents an undesired prolonged lifespan for NBs as occurs, for example, in the absence of Cas or Svp, when temporal scheduling is halted and prolonged NB lifespans result (Maurange et al. 2008). Conversely, the second group acts to safeguard NB cell fate and avoids early NB termination. Lack of these latter factors leads to the premature NB decommission and results in a shortened NB lifespans. Grainyhead (Grh) is an example of latter group (Cenci and Gould 2005; Bakshi et al. 2020).

Nuclear Pros is considered as an indicator of the end of the NB lifespans (Li and Vaessin 2000; Cenci and Gould 2005; Maurange et al. 2008; Chai et al. 2013; Wu *et al.* 2019; Sang *et al.* 2022). During NB asymmetric divisions, Pros is always cytoplasmic and is segregated exclusively into ganglion mother cells (GMCs) after each round of cell division (Hirata et al. 1995; Spana and Doe 1995; Ikeshima-Kataoka et al. 1997). Whenever nuclear Pros is observed in NBs, the consensus is that these cells will soon undergo terminal symmetric division and terminate their NB cell fate (Li and Vaessin 2000; Lai and Doe 2014). Thus, one of the important roles for the maintenance of NB proliferation is to prevent the premature accumulation of Pros in the nuclei of NBs. Although it has been reported that RanGAP, a nucleocytoplasmic transport regulator, is involved in Pros accumulation in the nuclei (Wu *et al.* 2019), the detailed regulatory mechanism of how NBs prevent premature nuclear Pros remains largely unknown.

The pipsqueak domain family genes are conserved between invertebrate and vertebrate species (Siegmund and Lehmann 2002). They contain DNA binding motifs and regulate genes expression. Two pipsqueak domain gene family members in *Drosophila, distal antenna* (*dan*) and *distal antenna-related* (*danr*) (Emerald *et al.* 2003) are in particular focus in this study. Previous works have documented that they share a large proportion of protein sequences and exhibit redundant functions (Siegmund and Lehmann 2002; Emerald *et al.* 2003; Suzanne et al. 2003; Kohwi et al. 2011). During the *Drosophila* embryonic neurogenesis, Dan and Danr (Dan/Danr) regulate Hunchback (Hb) expression (Kohwi et al. 2011). In the development of both eyes and antenna they are known to be involved in transcription regulation networks (Emerald *et al.* 2003; Suzanne et al. 2003).

In this study, we uncover a novel function for Dan/Danr relating to NBs cell fate maintenance in *Drosophila* third instar larval brains in which Dan/Danr act to prevent early accumulation of Pros in NB nuclei. Lack of Dan/Danr causes nuclear Pros accumulation in NBs which results in defective NB cell cycles and loss of the NB markers Ase and Dpn. Importantly, Removal of Pros from *dan/danr* MARCM clones is able to prevent NB marker loss and small lineage phenotypes. It appears that Dan/Danr and nuclear Pros are mutually exclusive in the third instar larval brain NBs. Meanwhile, Dan/Danr are partially required for Grh expression. Our work provides strong evidence that Dan/Danr antagonizes the function of nuclear Pros to maintain NB cell fates in third instar larval brains.

Results

dan/danr are required for NB proliferation

dan and *danr* are *Drosophila* pipsqueak domain family genes and share highly conserved protein sequences (Emerald *et al.* 2003). According to Flybase (www.flybase.org), they are mainly expressed in the larval central nervous system (CNS). To explore the function of *dan/danr* in CNS development in larval brains, we generated *dan/danr* MARCM clones in NBs. Since type I NBs represent the majority of NBs in the central brain, we only focused on type I NB clones. We found the mutant NB lineages to be smaller in size than those of controls (Figure 1A-B). In addition, on average each *dan/danr* clone contained only 20 ± 7 (n = 13) cells whereas approximately 85 ± 13 (n = 14) cells were found in the controls at 96 hr ALH (Figure 1C).

Meanwhile, we generated the antibody and performed immunostaining to show the dynamic expression pattern of Dan/Danr. The fusion protein containing the full length of the amino acid sequence of Dan was used as the antigen to raise the antibodies. The antibodies recognized both Dan and Danr proteins due to their sequence similarities (Supplemental figure 1A - D). Anti-Dan/Danr staining showed that they are highly expressed in NBs of the third instar larval brains (Figure 1D).

The *dan/danr* mutant line used in our MARCM clone experiments carried a deletion uncovered only three genes: *dan*, *danr* and *lobo* (Emerald *et al.* 2003; Kohwi *et al.* 2011). It has been previously reported that Dan and Danr exhibit redundant functions and that *lobo* is not expressed in the CNS. Meanwhile, animals with homozygous *lobo* are viable (Kohwi *et al.* 2011; Yang *et al.* 2011). No obvious NB defects were observed in the third instar larval brains upon single mutations of *dan*, *danr* or *lobo*.

Based on these observations, the *dan*/*danr* mutation is obviously responsible for the NB proliferation defects in the MARCM experiments. Thus we conclude that *dan/danr* are required for NB proliferation in the larval brains.

Lack of *dan/danr* leads to nuclear Pros in NBs

The observation of smaller lineages of *dan/danr* NB clones prompted us to examine Pros location in these NBs since nuclear Pros has been considered as a typical signal for the termination of NB cell fate

(Lai and Doe 2014; Wu *et al.* 2019; Sang *et al.* 2022). Nuclear Pros was indeed observed in the *dan/danr* NBs (Figures 2A - C). Approximately 61 % (n = 92) of the NBs in *dan/danr* MARCM clones presented with nuclear Pros at 96 hr ALH, whilst no corresponding presence of Pros was observed in any of their counterparts in *wt* controls (n = 30). These observations suggest that lack of *dan/danr* leads to nuclear Pros accumulation in larval brain NBs.

To examine the time window of the appearance of nuclear Pros in *dan/danr* NB clones, NBs were checked at different development stages. *dan/danr* clones showed nuclear Pros in the NBs between 72 hr to 120 hr ALH, with a peak at 96 hr ALH (Figure 2C). The frequencies of NBs with nuclear Pros were 28 % (n = 60) at 72 hr ALH, 61 % (n = 92) at 96 hr ALH, and 26 % (n = 69) at 120 hr ALH (Figure 2C). No NBs with nuclear Pros were observed in *wt* controls among these time periods. Based on those data we propose that Dan/Danr act to prevent the appearance of nuclear Pros between 72 hr to 120 hr ALH.

Taken together, the appearance of nuclear Pros in *dan/danr* NBs from 72 hr to 120 hr ALH suggests that the lack of *dan/danr* results in nuclear Pros in third instar larval brain NBs.

Mutation of *dan/danr* alters the NB cell cycle

Previous studies have reported that NBs with nuclear Pros often exhibit cell cycle defects (Li and Vaessin 2000; Wu *et al.* 2019; Liu *et al.* 2020). To determine the cell cycle progression of *dan/danr* NBs, we employed the Edu incorporation method and found that less NBs were Edu positive in *dan/danr* clones as compared with the controls at 96 hr ALH (Figures 3A - E). Only 35 % (n = 99) of NBs were labeled by Edu in *dan/danr* clones whereas the ratio of Edu positive NBs was almost 70 % (n = 91) in the controls (Figure 3E). We noted that at 72 hr ALH, the ratio of Edu positive NBs was also lower in *dan/danr* clones, being about 41 % (n = 56) compared to 68 % (n = 51) in the controls (Figure 3E). At 120 hr ALH, it was only 7 % (n = 92) in *dan/danr* clones but 82 % (n = 102) in the controls (Figure 3E). It appears that in the clones the ratio of Edu positive NBs was decreasing along with development, which was completely different from that of *wt* counterparts.

To further explore the cell cycle status of *dan/danr* NBs we performed immunofluorescence staining with another cell cycle marker, PH3, at different developmental stages (Figures 3F - J). Unexpectedly, we found that more NBs (71 %, n = 95) were PH3 positive in *dan/danr* clones as compared with the controls (35 %, n = 90) at 96 hr ALH (Figure 3J). We also checked the NBs at 72 hr and 120 hr ALH (Figure 3J). The results showed that the PH3 positive frequencies were also higher in *dan/danr* NBs being 46 % (n = 95) at 72 hr ALH, and 52 % (n = 92) at 120 hr ALH in *dan/danr* clones compared to 42 % (n = 90) at 72 hr ALH and 35 % (n = 101) at 120 hr ALH) in the controls. This result suggests that more *dan/danr* NBs remain within the cell cycle.

The observations of lower ratio of Edu positive and higher proportions of PH3 positive *dan/danr* NBs suggest that in the absence of Dan/Danr, the S-phase time is proportionally shortened while the M-phase time is lengthened within a single cell cycle, leading to an overall elongated cycle. Thus, Dan/Danr are also involved in NB cell cycle regulation.

The expressions of Ase and Dpn are inhibited in *dan/danr* NBs.

Reported NB terminating features include the presence of nuclear Pros and the loss of NB markers (Maurange *et al.* 2008; Chai *et al.* 2013; Wu *et al.* 2019). We have already shown that, in *dan/danr* clones, nuclear Pros appears in NBs. We further checked the cell fate identity of NBs using anti-Ase and anti-Dpn, two commonly used markers for type I NBs. The absence of either marker expression would indicate a change of NB cell fate. We first looked at Ase expression since all type I NBs were Ase positive. Assuming that all large cells (~10 mm) were NBs, we found that 11 % (n = 51) of the large cells in *dan/danr* MARCM clones away from the area where type II NBs were located were not labeled by Ase at 72 hr ALH (Figures 4A - D). This ratio further increased from 72 hr to 120 hr ALH (Figure 4D). About 15 % (n = 61) of NBs were shown to be Ase negative at around 96 hr ALH, with the percentage being 44 % at 120 hr AHL (n = 76) (Figure 4E). However, in *wt* controls all type I NBs were Ase positive (n = 30). This observation suggests that the NBs had gradually lost their NB identity in *dan/danr* mutant clones. Meanwhile, Dpn immunofluorescence staining showed a similar tendency in that the ratio of Dpn negative NBs increased from 72 hr ALH to 120 hr ALH in *dan/danr* clones (Figures 4A - C and 4E). This indicates that, in the absence of Dan/Danr, the NB cell fate gradually changes over time, as evidenced by the loss of Ase and Dpn expressions.

Nuclear Pros disrupts NB cell fate maintenance in *dan/danr* NBs

The loss of NB cell fates and appearance of nuclear Pros in *dan/danr* NBs led us to explore the interrelationship between these two events. We therefore double labeled NBs in *dan/danr* clones with Ase and Pros at different time points. The ratios of nuclear Pros and Ase double positive NBs increased from 72 hr to 96 hr ALH (Figures 5A - D), from 25 % (n = 57) to 58 % (n = 76), respectively. It seems that the appearance of nuclear Pros occurs earlier than the disappearance of Ase expression. Since nuclear Pros alone could inhibit Ase expression in NBs (Choksi *et al.* 2006; Lai and Doe 2014), it is likely that NBs with nuclear Pros will lose Ase expression at a later stage.

We then checked whether nuclear Pros suppressed Dpn expression. It is known that heat-shock induced overexpression of Pros in *wt* NBs leads to nuclear Pros accumulation (Choksi *et al.* 2006; Lai and Doe 2014). We observed that nuclear Pros from heatshock induced expression inhibited the stem cell marker Dpn expression in larval brain NBs (Figure 5E). This observation confirms that nuclear Pros alone is sufficient to suppress NB marker Dpn expression in NBs.

Based on these data we propose that the nuclear Pros in *dan/danr* NBs is most likely to be responsible for the altered expression of these two NB markers. To further confirm this hypothesis, we removed Pros from *dan/danr* NBs by producing a *pros/ dan/danr* triple mutant MARCM clone. We found that the Ase or Dpn expression phenotype in mutant NBs were rescued (Figure 5F-O). Ase immunofluorescence staining data showed that all NBs (n = 24) showed Ase positive in *pros/dan/danr* triple mutant clones, whereas there were about 82 % (n = 43) NBs showing Ase positive in their *dan/danr* double mutant counterparts (Figures 5F - I and 5J). Dpn staining also revealed similar results. All NBs (n = 30) exhibited Dpn expression in *pros/dan/danr* clones, while only 82 % (n = 37) NBs showed Dpn in the *dan/danr* NB clones

(Figures 5K - N and 5O). Furthermore, the clone sizes of *pros/dan/danr* NB were larger than those of *dan/danr* clones (Figure 5P - S). There were 32 ± 8 (n = 11) cells in each *pros/dan/danr* clone, whereas only 17 ± 8 (n = 9) cells in each of the *dan/danr* clones (p = 0.009) (Figure 5S).

Based on these data, nuclear Pros suppresses Ase and Dpn expression in *dan/danr* NBs and the removal of nuclear Pros rescues not only Ase and Dpn expression but also the NB lineage number. Thus, we conclude that nuclear Pros in *dan/danr* mutants are responsible for the disruption of NB cell fate maintenance.

Dan/Danr and nuclear Pros are mutually exclusive in NBs

Our data have shown that Dan/Danr act to prevent nuclear Pros formation in NBs. It would be interesting to understand the expression pattern of Dan/Danr at the early pupal stage when nuclear Pros appears in NBs prior to their termination. We firstly performed Dan/Danr and Pros double labeling in the *wt* third instar larval brains and found that Dan/Danr were present in the NB nuclei and that no nuclear Pros was detectable (Figure 6A). We then carried out Dan/Danr staining at late larval and early pupal stages. We found that at the early pupal stage (6 hr APF) the fluorescent signals of anti-Dan/Danr in NBs were weaker as compared with the ones at third instar larval stage (Figure 6B - C). And nuclear Pros was largely undetected even the cell sizes of NBs were smaller. In order to examine the expression pattern between Dan/Danr and Pros in terminating NBs, Dpn was used as NB marker and older pupal brains (16 hr APF) were adopted. Our Dpn, Dan/Danr and Pros triple-labeled experimental data showed that two groups of Dpn positive NBs were identified i) Dan/Danr positive, nuclear Pros negative, and ii) Dan/Danr negative, nuclear Pros positive (Figure 6D). Since these *wt* NBs were in a sequential process of NB termination, Dan/Danr negative/nuclear Pros positive NBs should represent the last state before terminal division. Thus, as Dan/Danr positive, nuclear Pros negative NBs are prior to Dan/Danr negative/nuclear Pros positive NBs, it is logical to deduct that Dan/Danr prevents nuclear Pros in NBs. At the time when Dan/Danr expression are lost, nuclear Pros appears in NBs, which leads to the termination of NBs.

To further confirm our deduction, we performed a Pros over-expression experiment in the *wt* third instar larval brains. Double-labeled experiments indicated that heat-shock induced Pros in NBs led to nuclear Pros and that Dan/Danr were not detectable in these NBs with nuclear Pros (Figure 6E). This suggested the nuclear Pros alone is sufficient to inhibit Dan/Danr expression.

These data suggest that Dan/Danr functions to prevent nuclear Pros accumulation in the third instar larval NBs. At early pupal stage Dan/Danr expression are decreased and nuclear Pros appears in NBs. In addition, nuclear Pros could further suppress Dan/Danr expression as a part of a feedback loop. Thus, Dan/Danr and Pros are mutually exclusive in NBs.

Dan/Danr are partially required for Grh expression in NBs cell size controlling

The observations that lack of Dan/Danr leads to the appearance of nuclear Pros and changes of NB cell fate between 72 hr to 120 hr ALH, seemed reminiscent of aspects of NB temporal regulation. We therefore

examined Dan/Danr expression dynamics and found that they were easily detected in the *wt* NBs between 72hr and 120hr ALH. Moreover, the aforementioned defective phenotypes in *dan/danr* NBs, including nuclear Pros appearance, cell cycle defects, and cell fate changes, all occurred from 72 hr ALH. These implied that Dan/Danr functions in NBs from 72 hr to 120 hr ALH.

Cas and Svp are early temporal regulators which are expressed before the third instar larval brain NBs (Jacob *et al.* 2008). We wanted to know whether *cas* or *svp* affects Dan/Danr expression. Immunofluorescence data showed that Dan/Danr protein levels did not change in *cas* or *svp* MARCM clone NBs (Supplemental Figure 2), which suggested that *dan/danr* expression did not depend on the early temporal regulators Cas and Svp.

It has also been reported that the temporal regulator Grh functions in the late stage of NBs temporal regulation (Cenci and Gould 2005; Chai *et al.* 2013). We checked *grh* expression in *dan/danr* NBs and found Grh expression was regulated by Dan/Danr (Figures 7A - C and 7D). Our fluorescence staining data showed that about 26 % (n = 66) of the *dan/danr* NBs failed to express Grh at 96 hr ALH (Figure 7D). Such data suggests that Dan/Danr are partially required for Grh expression in the third instar larval brain NBs.

We then examined the potential NB cell fate changes in the absence of Grh. The *grh* MARCM clones were induced in larval brains and we found that the cell sizes of NB became smaller and that Pros accumulated in NB nuclei (Figures 7E - J, and supplemental figure 3A-D). About 60 % (n = 84) of the NBs exhibited smaller cell sizes (< 8 mm) and about 29 % (n = 79) of the NBs showed nuclear Pros in *grh* MARCM clones at 96 hr ALH (Figure 7J and supplemental figure 3D). It seems clear therefore, that Grh prevents not only NB cell size shrinkage but also nuclear Pros accumulation in larval brain NBs.

In order to understand the relationship between Dan/Danr and Grh, we further quantified NB cell sizes in *dan/danr* NBs. Our data showed that about 24 % (n = 81) of NBs displayed smaller cell sizes (< 8 mm) in *dan/danr* clones at 96 hr ALH (Figure 7E - J). Since this ratio was similar to the percentage of NBs that had failed to express *grh* in *dan/danr* clones, it is possible that the loss of Grh in *dan/danr* NBs is responsible for the NB cell size change.

In conclusion, we believe the temporal factor Grh prevents cell size shrinkage and nuclear Pros formation in third instar larval brain NBs. It appears that Grh functions to control NB cell size is more pronounced. However, the specific mechanism of how Grh controls cell size remains unknown. Our data indicated that Dan/Danr were expressed in NBs between 72 hr to 120 hr ALH and that Grh fails to express in some of *dan/ danr* clones, which may then lead to the small NB cell size phenotype (< 8 mm).

Discussion

Sustained neural stem cell proliferation plays a vital role in neurogenesis. Our work shows that the *Drosophila* pipsqueak domain transcription factors Dan and Danr are required for NB cell fate maintenance.

The transcription factors Dan/Danr have been reported to be involved in the embryonic development of both the eye and the CNS (Emerald *et al.* 2003; Curtiss *et al.* 2007; Kohwi et al. 2011). Here we find that Dan/Danr are required for the larval brain NBs cell fate maintenance. Immunofluorescence staining shows that *dan/danr* are expressed in the larval brain NBs. In the absence of Dan/Danr, NBs produce less progenies and their lineages become smaller. This result indicates that the NB cell fate is altered due to loss of Dan/Danr. The *dan/danr* NBs exhibit nuclear Pros which then affects NB proliferative activity. Our data of the staining of the NB markers Ase and Dnp also shows a loss of the molecular features of *dan/danr* NBs. In addition, the cell cycles of *dan/danr* NBs are altered. Thus, we draw the conclusion that Dan/Danr are necessary for NB cell fate maintenance.

The absence of Dan/Danr leads to three events in the larval brain NBs: i) Pros accumulation in nuclei; ii) loss of NB markers (Ase and Dpn); and iii) cell cycle alteration. We believe that among these, NB molecular marker loss and cell cycle alteration are the consequence of the presence of nuclear Pros in *dan/danr* NBs. Our data support the hypothesis that, in the absence of Dan/Danr, Pros enters the nuclei and then suppresses the expression of NB markers Ase and Dpn. We have shown that Pros appears in the nuclei in the majority of NBs prior to Ase fade-away at 96 hr ALH. It has been already reported that heatshock induces the overexpression of Pros and that this results in nuclear Pros accumulation and the suppression of the expression of the NB markers Ase and Dpn (Choksi et al. 2006; Lai and Doe 2014). In addition, our data show that all NBs retain Dpn and Ase expressions when *pros* is removed from *dan/danr* clones. Furthermore, we found that the removal of *pros* in *dan/danr* NBs partially rescues the NB lineage size shrinkage phenotype. This indicates that nuclear Pros is also partially responsible for the cell cycle alteration in *dan/danr* NBs. This is consistent with the previous reports that Pros regulates not only NB marker expressions but also cell cycle progression (Li and Vaessin 2000; Lai and Doe 2014). Thus, Dan/Danr act to prevent premature nuclear Pros, cell cycle defects, and NB cell fate changes.

At the larval stage, Dan/Danr are mainly expressed in NBs while nuclear Pros remains undetected. At the early pupal stage, Dan/Danr are decreased in NBs, just at the time point that Pros signals begin to present themselves in the nuclei of the NBs. In our confocal images, Dan/Danr positive NBs failed to show nuclear Pros signals, and vice versa. Premature nuclear Pros is only detected in *dan/danr* mutant NBs and the overexpression of Pros in *wt* NBs results in the detection of nuclear Pros and suppression of Dan/Danr. These observations suggest that Dan/Danr and nuclear Pros appear to antagonize each other and are mutually exclusive in NBs.

In order to further uncover the relationship between Dan/Danr and nuclear Pros, we checked RanGAP expression in *dan/danr* NBs. It had previously been reported that a lack of RanGap facilitates nuclear Pros accumulation in NBs (Wu *et al.* 2019). However, we did not observe obvious changes in RanGAP staining patterns. This suggests that RanGAP is not regulated by Dan/Danr and that other pathway(s) must be involved in this process. In addition, *dan/danr* RNA-seq was also performed. Unfortunately, the data failed to lead to any useful conclusions. More work needs to be done to elucidate the specific relationship between Dan/Danr and nuclear Pros.

Grh is a late temporal regulatory factor. It prevents premature NB termination. In *grh* MARCM clones, about 60% of the NBs exhibited smaller cell sizes. We show that the Grh expression partially depends on Dan/Danr. In *dan/danr* clones, the ratio of *grh* negative NBs is about 26%. This coincides with the percentage of NBs displaying smaller cell sizes. This may reflect that Grh operates genetically downstream of Dan/Danr and acts to prevent NB cell size shrinkage.

Conclusion

In summary, our data demonstrate a novel function of pipsqueak domain transcription factors Dan/Danr in the third instar larval brain NBs (Fig. 8) where Dan/Danr act to maintain NB cell fates. In the absence of Dan/Danr, premature nuclear Pros appears, NB markers Ase and Dpn are suppressed, and cell cycle alteration is observed. In addition, Grh levels are downregulated which leads to smaller NB cell sizes.

Materials And Methods

Drosophila strains

All fly stocks and crosses were maintained at 25°C. We used the following *Drosophila* strains to analyze the phenotypes: $danr^{ex35}$, $dan/danr^{ex56}$, and dan^{emS3} (Emerald *et al.* 2003). We recombined $dan/danr^{ex56}$ together with *FRT* 82B to conduct MARCM analysis in the larval brains. Other mutant lines including: grh^{1M} *FRT* 42D / Cyo GFP, cas^{24} *FRT* 82B / TM6B, svp^{1} *FRT* 82B/TM6B (Chai *et al.* 2013) and $pros^{17}$ *FRT* 82B / TM6B (all shared by Dr. Cai) were used to analyze the NB clone phenotypes. The following Gal4 lines used to analyze the gene expression patterns or to overexpress the genes in NBs: *ase*-Gal4, *wor*-Gal4 (Bloomington stock center). The *UAS*-CD8::GFP line (THJ0080) was used to label the cells. The balancer lines used in this paper were $TM3^{sb}$/TM6B and Gla/Cyo (Bloomington stock center). *FRT* 42D and *FRT* 82B were used to induce clones in larval brains as controls.

Clonal analysis

MARCM clones were generated according to the standard method (Lee and Luo 2001). Once the cage had been set up, *Drosophila* embryos were collected over a period about 6 – 8 hr and then the embryos were kept at room temperature (RT). Heat-shock was conducted at around 24 - 30 hr after larval hatching (ALH) at 37°C in water bath for between 30 min to 1 hr as indicated. Dissection of the larval brains was conducted at the indicated time points (72 hr, 96 hr or 120 hr ALH) based on the corresponding genotypes, and then fixed for immunostaining.

Immunofluorescence staining and imaging

Brains were fixed 15 min at 4 % paraformaldehyde in 0.1 M HEPES pH 7.4. Antibody staining was performed according to the reported methods (An *et al.* 2017). The primary antibodies, dilutions, and sources used in this assay were: rabbit anti-DanDanr 1/1000; guinea-pig anti-DanDanr 1/1000; mouse anti-Pros 1/10; mouse anti-Elav (44C11) 1/10; rabbit anti-Caspase-3 1/1000 (Abcam); guinea anti-Dpn

1/1000 (Y. Cai’s lab); rabbit anti-Ase 1/1000; rabbit anti-Grh 1/1000 (Y. Cai’s lab); mouse, rabbit and Chicken anti-GFP (Abcam) and rabbit anti-phospho-histone H3 (PH3) (Abcam). Secondary antibodies were conjugated to either Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 633 (Molecular Probes), and used at 1/500, 1/1,000, or 1/250, respectively. TO-PRO-3 (Molecular Probes) at 1/5,000 was used for DNA staining and samples were mounted in Vectashield (Vector Laboratories). Images were obtained using OLMPUS upright microscope (FV-1000) and processed in Adobe Photoshop 2021. EdU incorporation was performed as per the kit instructions (Invitrogen).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated and analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HA, YX and YC conceived the idea of the project and designed the experiments. HA performed the experiments contributed to data analysis. YY, XR and MZ conducted the genetic experiments. FZ, HZ and RS generated the antibodies. YB and TL contributed to project discussion and coordination. HA wrote the initial manuscript. YX revised the manuscript. All authors read and approved the final manuscript.

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Figures

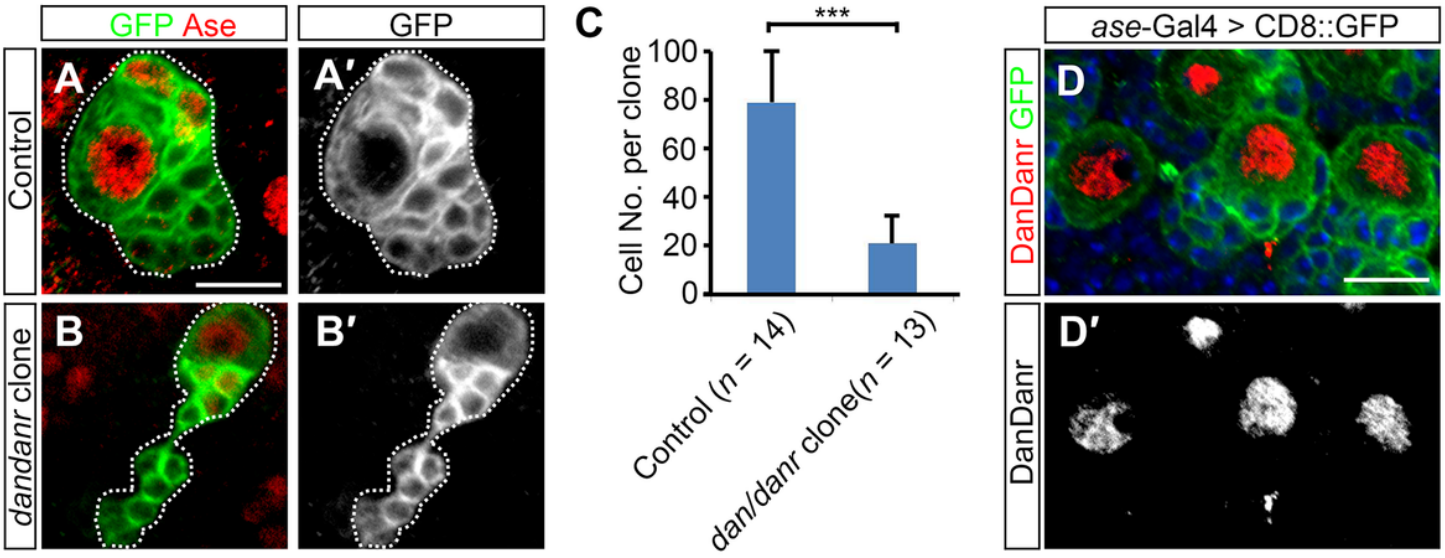

Figure 1

NB proliferation defects are found in *dan/danr* NBs.

(A-B') MARCM clones of type I NB labeled by GFP (green) in *Drosophila* third instar larval brains. Compared with controls (A-A'), the size of *dan/danr* MARCM clone (B-B') is smaller. NBs with Ase staining (red) are type I NBs. Dotted lines outline MARCM clones. **(C)** The statistical data of the average total cell number within a single lineage between control and *dan/danr* clones (***p value < 0.001). Only about a quarter of cells remains in *dan/danr* clones as compared with the controls. **(D-D')** Double labeling of anti-Dan/Danr (red) and GFP (green) in third instar larval brain NBs. GFP driven by *ase*-Gal4 marks type I NB lineages. Dan/Danr are expressed in type I NBs. Scale bars: 10 mm.

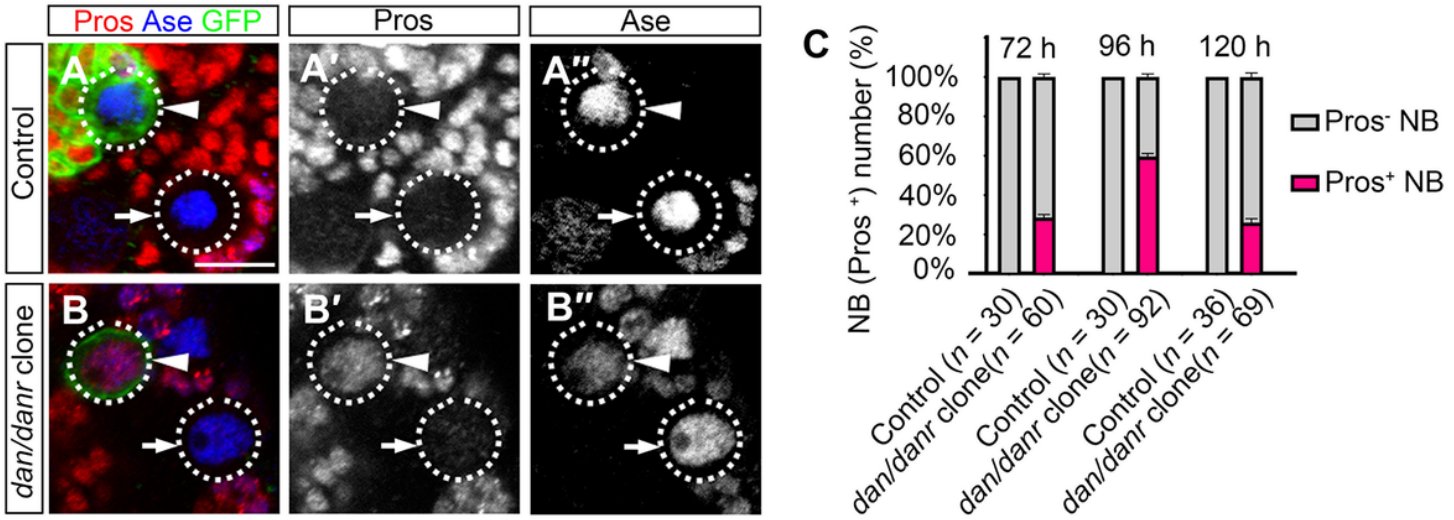

Figure 2

Loss of Dan/Danr leads to nuclear Pros in NBs.

(A-B') Anti-Pros and anti-Ase double labeling in third instar larval brains. (A-A") NBs in *wt* clone (arrowhead) and its neighborhood (arrow) fail to show Pros in nuclei. (B-B") NBs in *dan/danr* clones (arrowhead) exhibit nuclear Pros (red), but not in the *wt* NBs (arrow) in its neighborhood. This indicates that the loss of Dan/Danr is responsible for the nuclear Pros in NBs. GFP (green) labels the MARCM clones. Scale bar:10 mm. **(C)** The quantifications of larval brain NBs with nuclear Pros at 72 hr, 96 hr and 120 hr after larvae hatching (ALH). The percentage of NBs containing nuclear Pros is to about 60 % at 96 hr ALH.

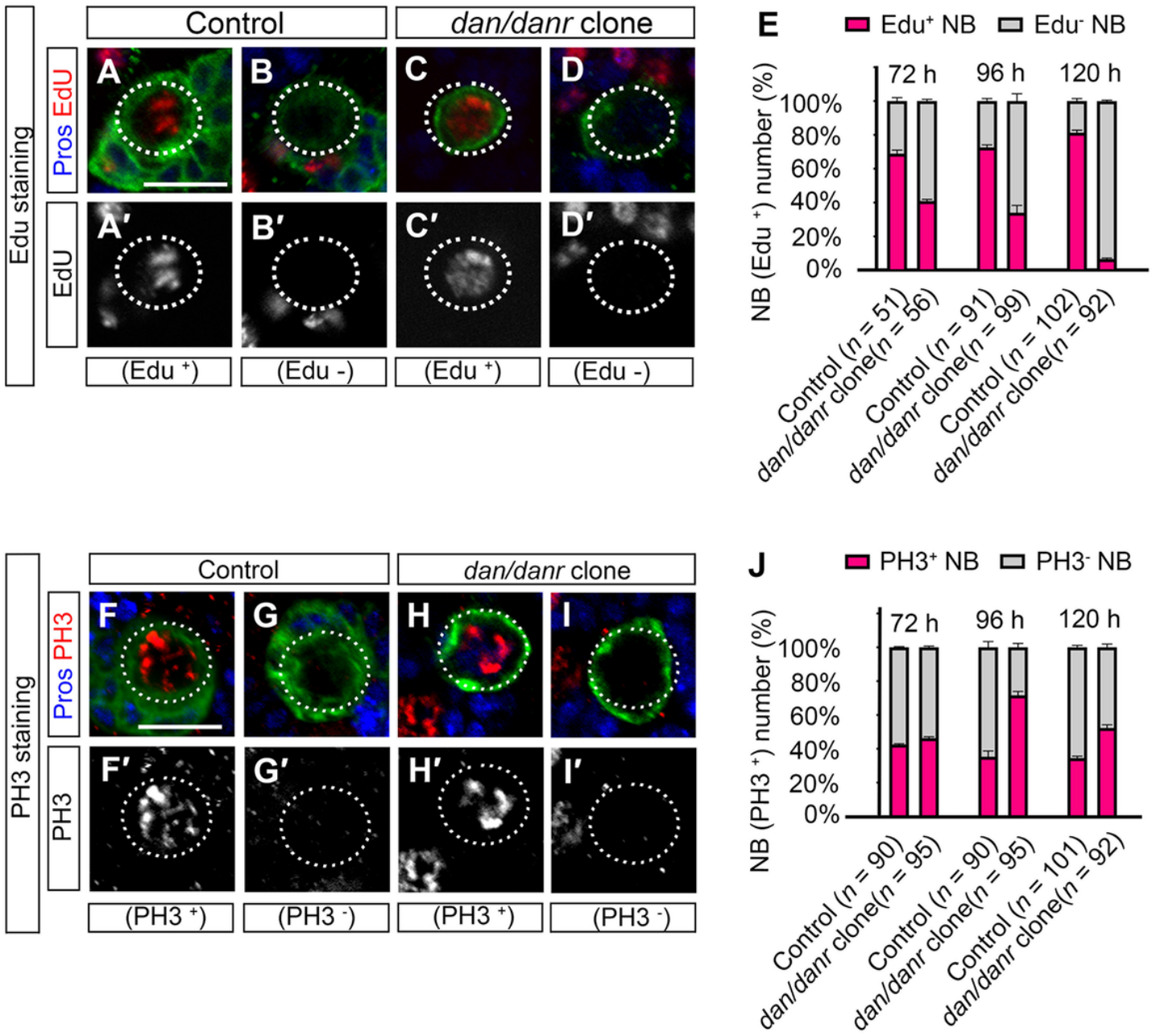

Figure 3

Mutations of *dan/danr* cause NB cell cycle defects in the third instar larval brains.

(A-D') Visualization of incorporated EdU in NBs of MARCM clones. EdU positive (red, A-A', C-C') and negative (B-B', D-D') NBs in the control and in *dan/danr* clones. **(E)** The quantitative data of Edu labeling in control and *dan/danr* NBs. The incorporation of EdU becomes less efficient with the time in *dan/danr* NBs. **(F-I')** Anti-PH3 staining of NBs in MARCM clones. PH3 (red, F-F', H-H') positive and negative (G-G', I-I') NBs in the control and *dan/danr* clones. **(J)** The quantitative data of PH3 positive NBs in control and *dan/danr* clones. The percentages of PH3 positive NBs in *dan/danr* clones are higher than those of the control NBs, which show the largest differences at 96 hr ALH. GFP (green) marks the MARCM clones. Dotted lines outline the NBs. Scale bars: 10 mm.

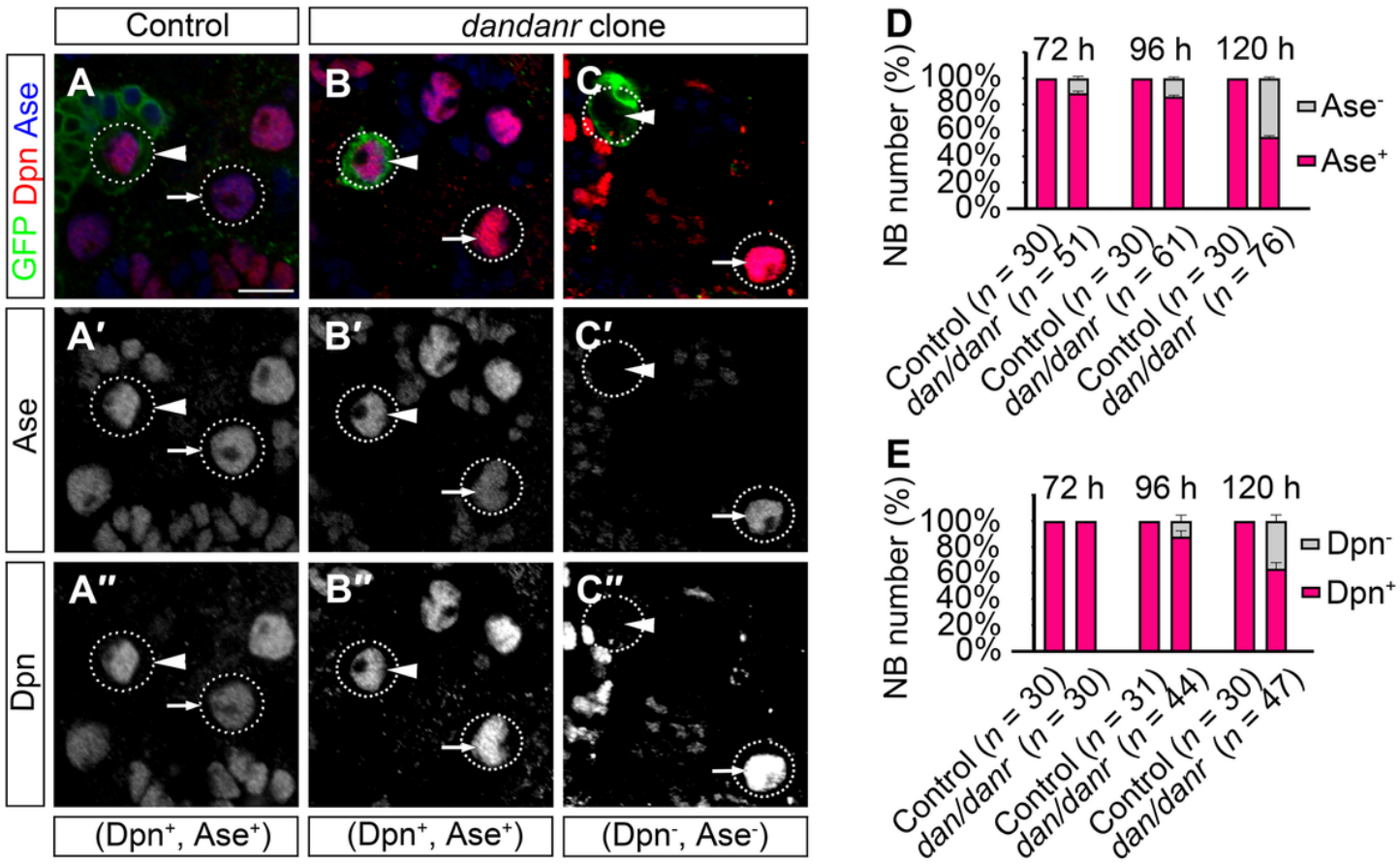

Figure 4

Expressions of Ase and Dpn are suppressed in *dan/danr* NBs.

(A-C") Anti-Ase (blue) and anti-Dpn (red) double labeling of NBs in MARCM clones in third instar larval brains. NBs in *wt* clone (arrowhead) and its neighbor (arrow) show as Ase and Dpn positive (A-A"), while NBs in *dan/danr* clones present with two types: Ase and Dpn positive NBs (arrowhead, B-B") or Ase and Dpn negative (arrowhead, C-C"). Note the *wt* NBs outside the clones (arrow, B-C") are Ase and Dpn positive. This suggests that the expressions of Ase and Dpn are suppressed in *dan/danr* NBs. GFP (green) marks the clones. Scale bar:10 mm. **(D-E)** The statistical data of Ase (D) and Dpn (E) expressions in NBs of both control and *dan/danr* clones at 72 hr, 96 hr and 120 hr ALH. In *dan/danr* MARCM clones, Ase and Dpn expressions are gradually inhibited in NBs. The ratio of NBs that are Ase and Dpn negative rises up to 40% in late larval brains (120 hr ALH).

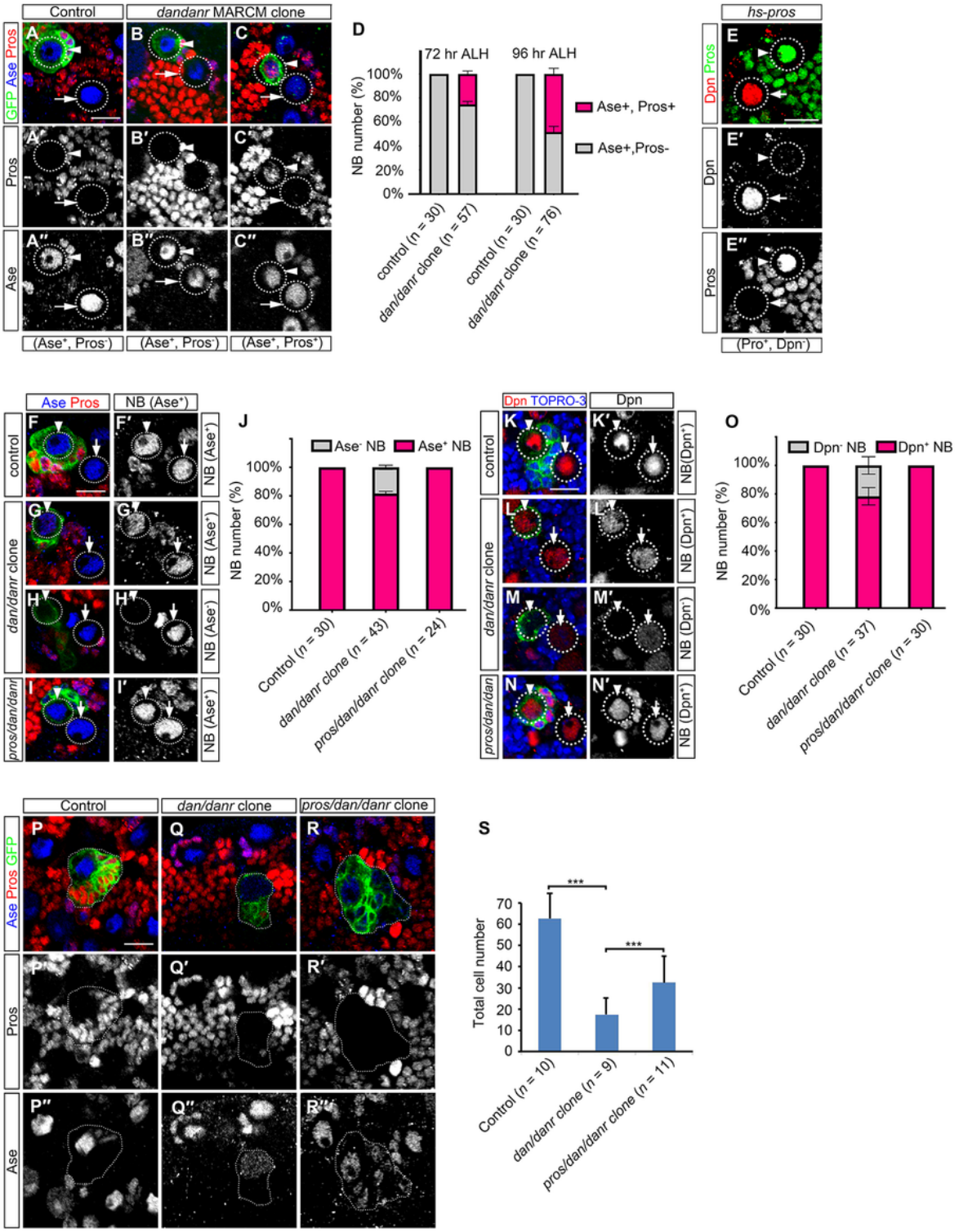

Figure 5

Nuclear Pros disrupts NB cell fates in *dan/danr* NBs in third instar larval brains.

(A-C") Anti-Pros (red) and anti-Ase (blue) double labeling of NBs in MARCM clones. (A-A") Type I NBs in control clones (arrowhead) and in the neighborhood (arrow) are Ase positive (blue) and nuclear Pros negative. (B - C") NBs in *dan/danr* clones show up in one of two groups: either Ase positive (blue) and

nuclear Pros negative (arrowhead, B-B"), or Ase positive (blue) and nuclear Pros positive (red) (arrowhead, C-C"). Note the *wt* NBs outside the clones (arrows, B-C") are Ase positive and nuclear Pros negative. **(D)** Statistical data shows both Ase and nuclear Pros positive NBs in control and *dan/danr* clones at 72 and 96 hr ALH. It seems that the appearance of nuclear Pros occurs earlier than the disappearance of Ase expression. **(E-E")** Anti-Pros (green) and anti-Dpn (red) double staining of the heat-shock treated NBs. Heat shock induced Pros causes nuclear Pros accumulation (green, E and E") and inhibits Dpn expression in NBs (arrowhead). The NBs (arrows) without nuclear Pros are Dpn positive (red, E and E'). This suggests nuclear Pros inhibits Dpn expression in NBs. **(F - I'):** Anti-Pros (red) and anti-Ase (blue) double labeling of the type I NBs in MARCM clones. (F-F') Type I NBs in *control* clones (arrowhead) and outside clones (arrow) show as Ase positive (blue). (G-H') Two types of NBs in *dan/danr* clones are identified: Ase positive (blue, arrowhead, G-G') or Ase negative (arrowhead, H-H'). (I-I') NBs in *pros/dan/danr* triple mutant clones (arrowhead) are Ase positive. Note the NBs outside the clones (arrow, G-I') are *wt.* **(J)** The statistical data of Ase positive NBs in different genotypic backgrounds (*control, dan/danr, pros/dan/danr*). In *dan/danr* clones about 20% NBs fail to show Ase expression. This phenotype is reversed in *pros/dan/danr* triple mutant clones, indicating that suppression of Ase is due to nuclear Pros in *dan/danr* NBs. **(K-N')**: Anti-Dpn (red) staining of the NBs. (K-K') NBs in *control* clones (arrowhead) and its neighbors (arrow) are Dpn positive. (L-M') Two types of NBs in *dan/danr* clones are observed: Dpn positive (red, arrowhead, L-L') and Dpn negative (arrowhead, M-M'). (N-N') NB in *pros/dan/danr* triple mutant clones (arrowhead) is Dpn positive. Please note that the NBs outside the clones (arrow, K-N') are *wt.* **(O)** Statistical data of Dpn negative NBs in different genotypes (*control, dan/danr, pros/dan/danr*). In *dan/danr* clones about 20% NBs fail to show Dpn expression and this phenotype is reversed in *pros/dan/danr* triple mutant clones. This indicates that, in a similar observation to Ase, suppression of Dpn is due to nuclear Pros in *dan/danr* NBs. **(P - R)** The sizes of MARCM clones of different genotypes (P: *control,* Q: *dan/danr,* R: *pros/dan/danr*). **(S)** The statistical data of average total cell numbers in clones of different genotypes (***P < 0.001). The *dan/danr* clone sizes are partially rescued by the removal of Pros (*pros/dan/danr*). GFP (green) marks MARCM clones. Circular dotted lines outline NBs. Dotted lines outline MARCM clones. TO-PRO-3 (Blue) labels DNA. Scale bars: 10 mm.

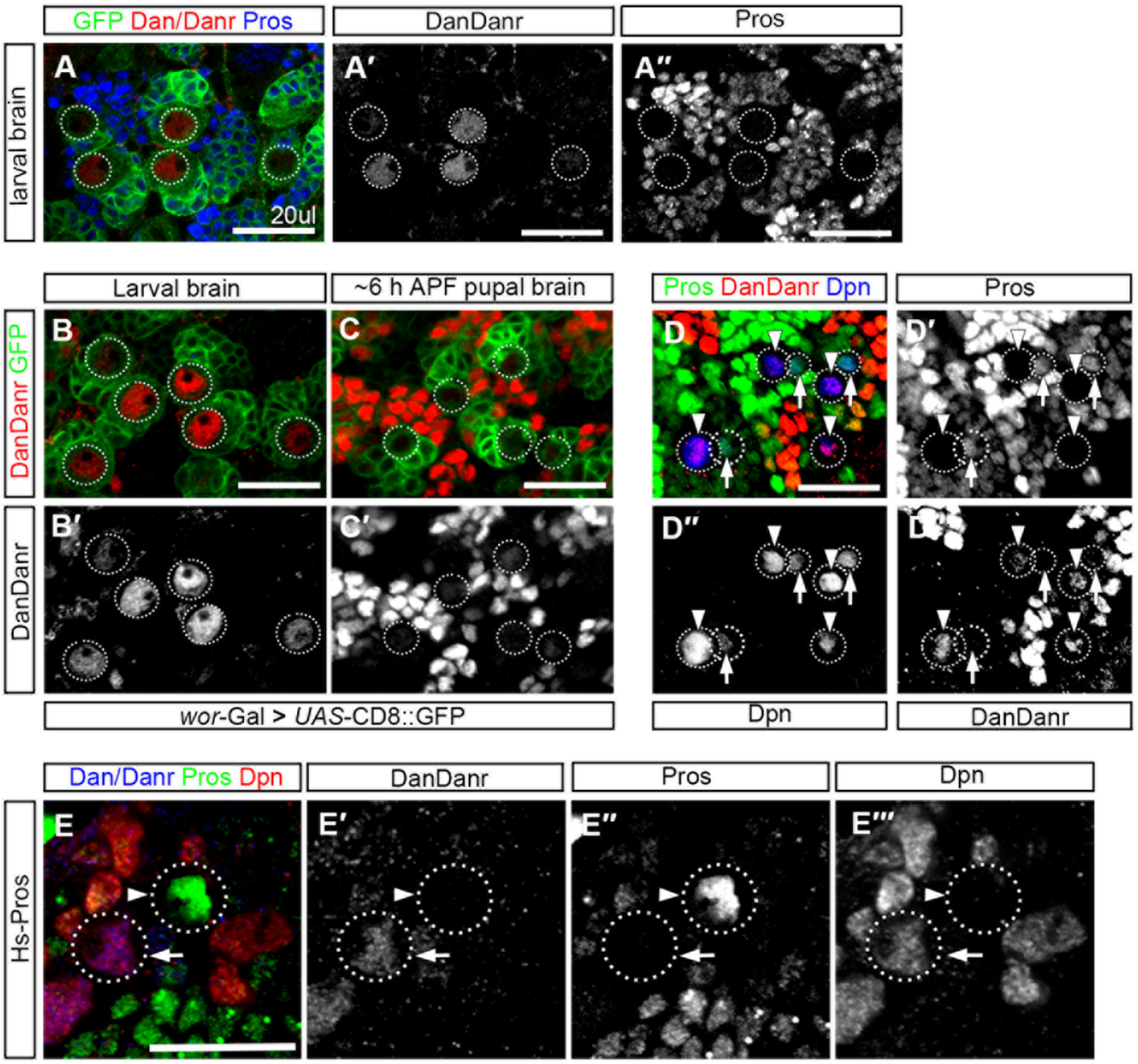

Figure 6

Dan/Danr and nuclear Pros are mutually exclusive in NBs.

(A - A") Anti-Dan/Danr and anti-Pros double labeling of the NBs in third instar larval brains. Dan/Danr (red, A and A') are detected in NBs, whilst nuclear Pros (blue, A and A") remains undetectable. Both of them do not coexist in NBs. GFP (green) derived by ase-Gal4 marks NBs and their progeny. Dotted lines outline the NBs. Scale bar: 20 mm. **(B - C')** Anti-Dan/Danr (red) staining of the NBs between larval to early pupal stage (6hr after pupae formation, APF). Dan/Danr is easily detected in larval brain NBs (red, B-B'), but is barely detected in pupal brain NBs (C-C'). This suggests that Dan/Danr expression level is decreased in pupal NBs. Note that NBs become smaller in early pupal brains. GFP (green) derived by *ase* - Gal4 marks

NBs and their progeny. Dotted lines outline the NBs. Scale bar: 20 mm. **(D - D‴)** Anti-Dpn (blue), anti-Dan/Danr (red) and anti-Pros (green) triple staining of NB in early pupal stage (~16 hr APF). Two type NBs are detected: Dpn+, Dan/Danr +, nuclear Pros – NBs (Arrowheads) and Dpn+, Dan/Danr -, nuclear Pros + NBs (Arrows). Dpn positive cells are NBs. Dotted lines outline the NBs. The scale: 20 mm. **(E - E‴)** Anti-Pros (green), anti-Dan/Danr (blue), and anti-Dpn (red), triple staining of the heat shock treated NBs. Heated shock induced Pros leads to nuclear Pros (arrowhead, green) in NBs and prevents Dan/Danr expression. The NBs without nuclear Pros show Dan/Danr (arrow, blue). This observation indicates that nuclear Pros and Dan/Danr are mutually exclusive in NBs. Dotted lines outline the NBs. Scale bar: 40 mm.

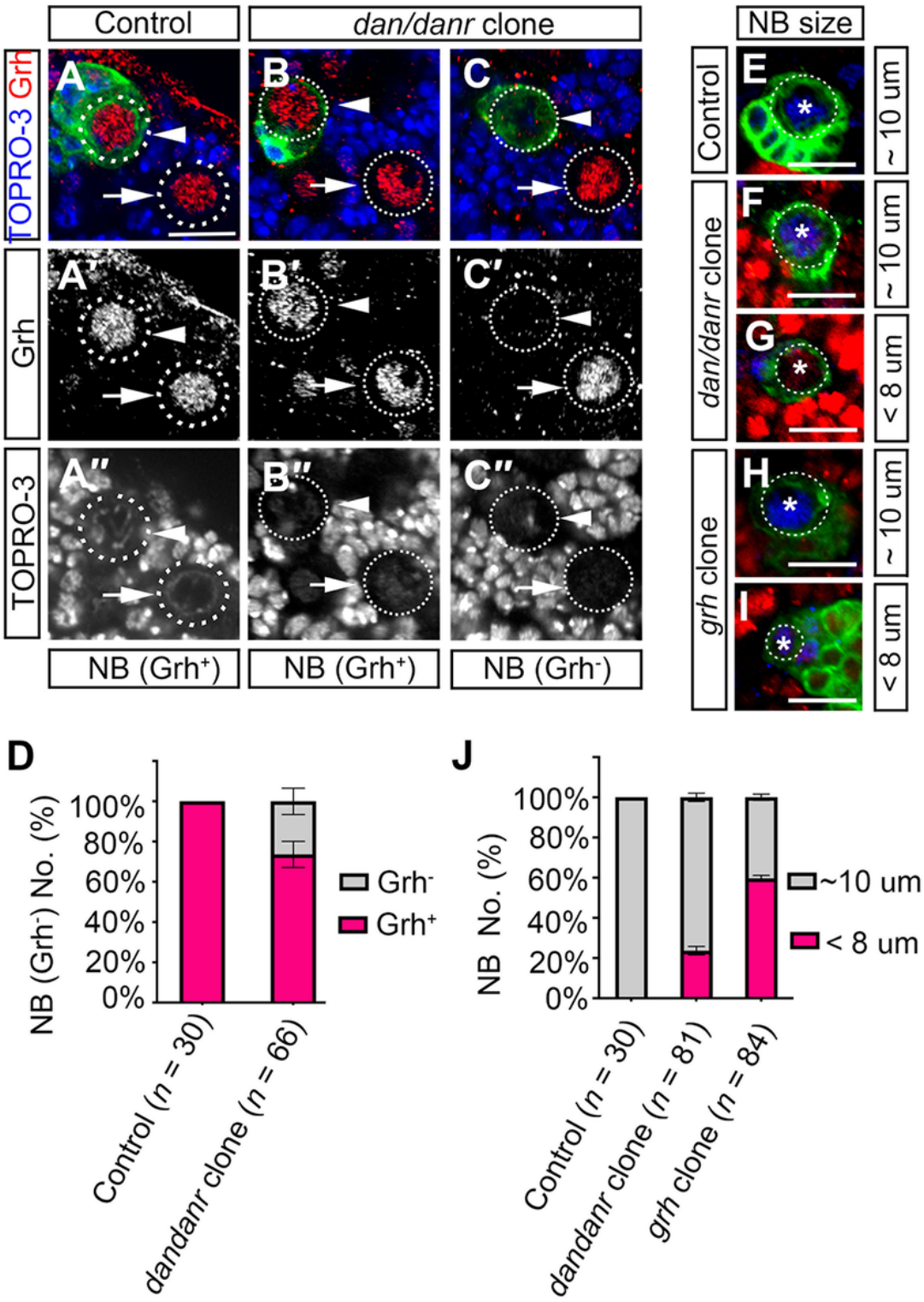

Figure 7

Dan/Danr are partially required for Grh expression in NBs cell size controlling.

(A-C") Anti-Grh (red) staining of NBs in *dan/danr* MARCM clones of third instar larval brains. (A-A") NBs in control clones (arrowhead) and in the neighborhood (arrow) show as Grh (red) positive. (B-C") In *dan/danr* clones, two types of NBs are detected: Grh (red) positive NBs (B-B", arrowhead) and Grh

negative NBs (C-C", arrowhead). Their neighboring NBs (arrow) are *wt* and show as Grh (red) positive. GFP (green) labels the clones and TO-PRO-3 (blue) marks the DNA. Scale bar: 20 mm. **(D)** The statistical data of Grh negative NBs in *dan/danr* clones at 96 hr ALH. About 20% of *dan/danr* NBs are Grh negative. This indicates that Dan/Danr are partially required for Grh expression in NBs. **(E - I)** The NBs cell sizes in *dan/danr* and *grh* MARCM clones at late larval stage. GFP (green) labels the control (E), *dan/danr* (F - G) and *grh* (H - I) clones. Ase (blue) marks the NBs. In control clones, the NB was around 10 mm. But in both of *dan/danr* and *grh*, smaller (< 8 mm) NBs are observed (G and I). Scale bars: 10 mm. **(J)** The statistical data of NBs cell sizes in control, *dan/danr* and *grh* clones at 96 hr ALH. It is possible that the loss of Grh expression in *dan/danr* NBs leads to smaller NBs cell sizes.

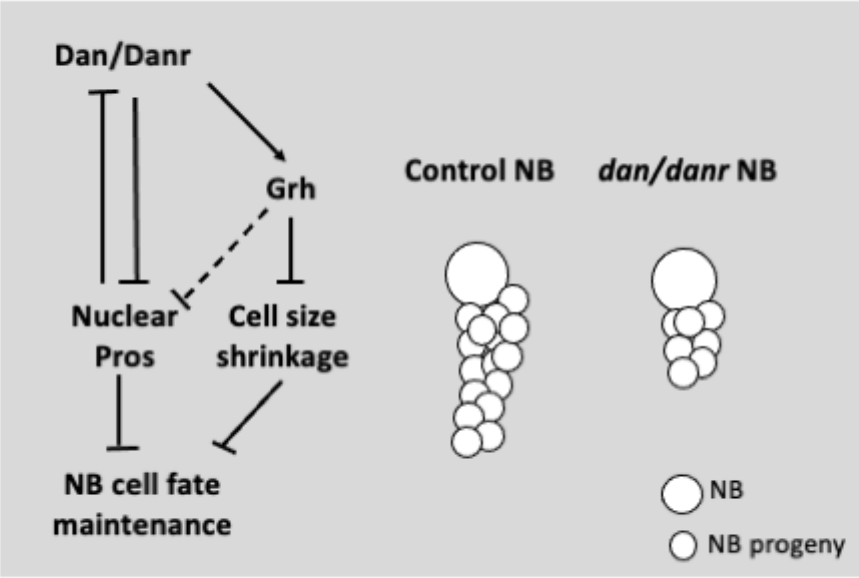

Figure 8

Diagram depicting Dan/Danr function. Dan/Danr antagonizes nuclear Pros to maintain NB cell fate in *Drosophila* third instar larval brains. During larval brain development, NBs maintain precise cell fate and size to form the central nervous system. In this process, *Drosophila* pipsqueak domain transcription factors, Dan/Danr act to antagonizes nuclear Pros in NBs and maintain NB cell fate. Dan/Danr are partially required for Grh expression to prevent the shrinkage of NB cell sizes. In addition, lack of Dan/Danr causes defective NB cell cycle progression, resulting in less progeny cells to result from NBs.

Supplementary Files

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- Supplementalmaterials.pdf