RESEARCH ARTICLE

Dachshund acts with Abdominal-B to trigger programmed cell death in the Drosophila central nervous system at the frontiers of Abd-B expression

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Abstract

A striking feature of the nervous system pertains to the appearance of different neural cell subtypes at different axial levels. Studies in the Drosophila central nervous system reveal that one mechanism underlying such segmental differences pertains to the segment-specific removal of cells by programmed cell death (PCD). One group of genes involved in segment-specific PCD is the Hox homeotic genes. However, while segment-specific PCD is highly precise, Hox gene expression is evident in gradients, raising the issue of how the Hox gene function is precisely gated to trigger PCD in specific segments at the outer limits of Hox expression. The Drosophila Va neurons are initially generated in all nerve cord segments but removed by PCD in posterior segments. Va PCD is triggered by the posteriorly expressed Hox gene Abdominal-B (Abd-B). However, Va PCD is highly reproducible despite exceedingly weak Abd-B expression in the anterior frontiers of its expression. Here, we found that the transcriptional cofactor Dachshund supports Abd-B-mediated PCD in its anterior domain. In vivo bimolecular fluorescence complementation analysis lends support to the idea that the Dachshund/Abd-B interplay may involve physical interactions. These findings provide an example of how combinatorial codes of transcription factors ensure precision in Hox-mediated PCD in specific segments at the outer limits of Hox expression.

KEYWORDS

cell diversity, Drosophila, Hox frontier assistant, hox genes, neuronal subtype specification, terminal differentiation
1 | INTRODUCTION

A fascinating property of many, if not all, nervous systems pertains to the region-specific appearance of distinct neuronal subtypes. Studies of the Drosophila developing central nervous system (CNS) have revealed that this feature can emerge from at least four different underlying mechanisms. First, the generation of different repertoires of progenitor cells, denoted neuroblasts (NBs) in Drosophila, in different segments (Birkholz et al., 2016; Birkholz et al., 2013; Urbach & Technau, 2004). Second, the development of segment-specific NB lineage size differences, whereby equivalent NBs in different segments generate differently sized lineages (Karlsson et al., 2010; Monedero Cobeta et al., 2017; Schmid et al., 1999; Schmidt et al., 1997). Such cell number differences may emerge from, for example, changes in the number of NB and/or daughter cell divisions in different segments (Monedero Cobeta et al., 2017; Yaghmaeian Salmani et al., 2018).

Third, segment-specific mechanisms can act to specify different neuronal subtype cell fates in different segments (Karlsson et al., 2010; Stratmann & Thor, 2017; Stratmann et al., 2019). Finally, cells that are initially generated throughout the neuro-axes may undergo segment-specific programmed cell death (PCD) (Gabilondo et al., 2011, 2018; Miguel-Aliaga & Thor, 2004, 2009; Rogulja-Ortmann et al., 2008; Suska et al., 2011).

Previous studies have demonstrated that the Hox homeotic genes play key roles during all four types of neurogenesis events (Arya & White, 2015; Merabet et al., 2011; Rogulja-Ortmann & Technau, 2008). However, our understanding of Hox gene involvement in these processes is still rudimentary. This particularly pertains to the contrast between the graded expression of Hox genes and proteins on the one hand and the precise segment-specific PCD of particular neurons on the other. One salient example of this is the role of the Abdominal-B (Abd-B) gene and the PCD of the Va neuropeptide expressing cells in the Drosophila ventral nerve cord (VNC). The Va neurons are initially generated throughout the Drosophila VNC, that is, in segments T1–A7, but display four segment-specific developmental fates (Gabilondo et al., 2011; Suska et al., 2011). In T1–T3, Va cells differentiate into an unknown cell type (Va-T), while in A1 they express the neuropeptides diuretic hormone 31 (DH31) and Allatostatin A (AstA; Va-DH31) (Gabilondo et al., 2018), in A2–A4 they express the Capability (Capa) neuropeptide (Va-Capa), and in A5–A7 they neurons undergo PCD by stage 16 (St16; Va-PCD) (Gabilondo et al., 2011, 2018; Suska et al., 2011). The Va neurons in all segments express the neuropeptide cell fate determinant Dimmed (Dimm), a bHLH transcription factor, while A1–A7 Va neurons also express the transcriptional cofactor Dachshund (Dac) (Figure 1a) (Miguel-Aliaga et al., 2004). Dimm controls the expression of many neuropeptides and peptide processing enzymes (Allan et al., 2005; Baumgardt et al., 2007; Hewes et al., 2003; Park et al., 2011; Stratmann et al., 2019). Dac acts in a combinatorial manner to dictate the neuropeptide cell fate of other neuropeptide neurons (Baumgardt et al., 2007; Miguel-Aliaga et al., 2004), but its role in Va development is unknown. The segment-specific differentiation/PCD of Va neurons has been found to be under the control of homeotic Hox genes. This has revealed that the thoracic Hox gene Antennapedia (Antp) promotes Va-T, Ultrabithorax (Ubx) promotes Va-DH31 fate, Abdominal-A (Abd-A) promotes Va-Capa fate, and Abdominal-B (Abd-B) promotes Va-PCD fate (Gabilondo et al., 2011, 2018; Suska et al., 2011) (Figure 1a). Previous studies have revealed that Abd-B is also sufficient to trigger PCD in anterior Va cells (Suska et al., 2011). In line with its role in Va PCD, previous studies have shown that Abd-B is expressed in Va neurons in A5–A7 (Suska et al., 2011). However, Abd-B is expressed in a gradient in the posterior abdominal segments (Hirth et al., 1998; Karlsson et al., 2010; Miguel-Aliaga & Thor, 2004) and is barely detectable by antibodies in A5–A6 Va neurons (Suska et al., 2011) (herein). Hence, Abd-B can trigger PCD in a highly reproducible manner in all four abdominal segments (A5–A7), despite being expressed at very low levels in A5–A6. This raises the question of how the action of Abd-B in the PCD of Va neurons in A5–A6 can be so stereotypically executed despite the weak expression of Abd-B toward its anterior extent of expression.

By conducting a targeted genetic screen, we found that dac, which encodes a transcriptional cofactor, is necessary for PCD in posterior Va neurons, hence mimicking the role of Abd-B in these neurons. dac and Abd-B do not regulate each other, and bimolecular fluorescence complementation (BiFC) analysis indicates that Dac and Abd-B physically interact inside Va neurons. These studies indicate that Dac acts as a cofactor for Abd-B activity, supporting its role as a proapoptotic determinant at the outer limits of Abd-B expression. We propose the term Hox frontier assistant (HFA) to describe the role of dac in this context. These findings may provide guidance for other events where Hox factors act in a context-dependent and low-abundance manner.

2 | MATERIALS AND METHODS

2.1 | Drosophila melanogaster stocks

Drosophila melanogaster stocks were raised, and crosses were performed at +25˚C on standard medium. The following stocks were used: elavC155 = elav-Gal4 (DiAntonio et al., 2001). Va-Gal4 (Allan et al., 2003). UAS-dac
Figure 1  *dac* is necessary but not sufficient for PCD in Va neurons. (a) Cartoon depicting the Va cells along the Drosophila VNC and their expression of Dimm, Dac, DH31, and Capa. (b–d) Staining for Capa in the developing VNC at 18hAEL, in control, *dac*3, and *elav-Gal4/UAS-dac* embryos. (e) Quantification of Va cells per VNC hemicord at 18hAEL (asterisk denotes $p \leq 0.05$; Student’s *t* test; mean ± SEM; $n > 10$ VNCs per genotype). (Shen & Mardon, 1997). *UAS-reaper* (Zhou et al., 1997). *UAS-AbdBm* and *FRT82B-AbdB*018 (obtained from E. Sanchez-Herrero). *Att*1938, *Bbg*9, *brut*55871, *dac*3, *dra*P241, *Dscam*05518, *dsh*75, *faj*BX4, *fra*2, *Fur*1P127, *Hr*802306, *hth*SE04, *kay*1, *msk*B185, *par*2, *qsm*05510, *rg*102605, *rhea*1, *rr*5, *run*2, *SoxN*NC14, *sta*r39, *tor*2, *tsh*5, *wts*1 (Bloomington Drosophila Stock Center). Mutants were kept over *CyO, Dfd-EYFP* or *TM6, Sb, Tb, Dfd-EYFP* balancer chromosomes. As wild type, *Oregon-R* was often used.

2.2 Fusion protein constructs and transgenic lines

For the BiFC assay, the UAS-dac-VN (173) construct was generated by polymerase chain reaction from a *dac* cDNA (provided by G. Mardon, Baylor College of Medicine, Houston, Texas, USA) with forward primer 5′-CAG TCT CGA GGG CCG CTC AGG CCG CAT GGA TTC TG TGA CAA GTG AAC-3′ and reverse primer 5′-CAG TCT CGA GGG CCG CTC AGG CCG CA TGG ATT CTG TGA CAA GTG AAC-3′, introducing flanking restriction sites (restriction sites in bold) and a linker of five amino acids to separate the Venus fragment from the protein of interest. Fragments were cloned into the XhoI-XbaI sites of a VN173 pUASTattB vector (Hudry et al., 2011). Transgenic flies were obtained by injecting the plasmid into *yw, M [3xP3-RFP.attP]ZH-86Fb* embryos (Bischof et al., 2007). The constructs were sequence-verified before fly transformation, and transgenic lines were established according to standard genetic practice. The *UAS-AbdB*VC line (Bischof et al., 2018) was provided by Samir Merabet (Institut de Génomique Fonctionnelle de Lyon, Lyon, France).

2.3 Immunohistochemistry

The antibodies used were rabbit α-Capa (1:1,000) (provided by J. Veenstra), mAb α-Dac (1:25) and mAb α-Abd-B (1:50) (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA; guinea pig α-Dimm (1:200) (Baumgardt et al., 2007). All polyclonal sera were preabsorbed against pools of early embryos. Secondary antibodies were conjugated with fluorescein isothiocyanate (FITC), rhodamine-RedX or Cy5 and used at 1:500 (Jackson ImmunoResearch, PA, USA). Embryos were dissected in phosphate-buffered saline (PBS), fixed for 25 min in 4% paraformaldehyde (PFA), blocked and processed with antibodies in PBS with 0.2% Triton X-100 and 4% donkey serum. Slides were mounted with Vectashield (Vector, Burlingame, CA, USA). In the cases where immunostaining levels were scored, control and mutant embryos were stained and analyzed on the same slide.

2.4 Confocal imaging, data acquisition, and staining quantification

A Zeiss META 510 confocal microscope was used to collect data for all fluorescent images; confocal stacks were merged using LSM software or Adobe Photoshop CS4. Where appropriate, images were false colored to assist color-blind readers or to represent the data more clearly.

2.5 Statistical methods

Statistical analysis was performed using Microsoft Excel. Quantifications of observed phenotypes were performed using Student’s two-tailed *t* test, assuming equal variance.
Table 1  Ectopic CAPA expression in A5–A7 abdominal segments in different mutant backgrounds

| Genotype | CAPA A5–A7 |
|----------|------------|
| Atu S1 938 | –          |
| Bgb9     | –          |
| bru3 05871 | –         |
| Chi e5.5 | –          |
| ci 94    | –          |
| CtBP 03463 | –         |
| dac³     | +          |
| Fur1 P127 | –          |
| Hr38 02306 | –        |
| bth SE04 | –          |
| kay¹     | –          |
| Mfap1 DsRed | –       |
| msk B185 | –          |
| NorpA 36  | –          |
| pan²     | –          |
| qsm 05510 | –          |
| rgr k02605 | –         |
| rhea¹    | –          |
| rn³      | –          |
| run³     | –          |
| SoxN NC14 | –          |
| stau r59  | –          |
| tou²     | –          |
| tsh8     | –          |
| wts¹¹    | –          |

Figure 2 dac acts upstream of rpr to drive PCD in Va neurons. (a, b) Staining for Capa and Dimm in the developing VNC at 18hAEL, in control and dac³; Va-Gal4/UAS-rpr embryos. (c) Quantification of Va cells per VNC hemicord at 18hAEL (asterisk denotes p ≤.05; Student’s t test; mean ± SEM; n > 10 VNCs per genotype).

Results

3.1 Dachshund is required for PCD of Va neurons within A5–A6

To shed more light upon the mechanisms by which PCD is secured in Va neurons at the outer limits of expression of the Abd-B gene, we conducted an exploratory screen of 25 genes known to be expressed in the CNS at embryonic stages 11–16 (based upon the Berkeley Drosophila Genome Project in situ atlas), scoring for mutants displaying ectopic Capa expression in the A5–A7 abdominal segments, which would indicate a failure in the PCD process (Methods; Table 1). In this screen, we identified that dac mutants display a failure in the PCD of Va neurons in the A5–A6 segments, by 18hAEL, a stage at which they normally have undergone PCD (Figure 1b,c,e and Supplemental Figure S3). Thus, dac is necessary for PCD in the Va neurons in A5–A6.

Misexpression of Abd-B in anterior segments is sufficient to trigger PCD of all Va cells (Suska et al., 2011). To address whether dac is also sufficient to induce Va PCD, we analyzed the effects of dac misexpression driven by the pan-neural elav-Gal4 driver (elav-Gal4 > UAS-dac). However, we found that the Va-Capa neurons in A2–A4 were unaffected by dac misexpression (Figure 1d,e and Supplemental Figure S4). Therefore, dac is necessary but not sufficient to trigger PCD in Va cells.

3.2 dac acts upstream of the proapoptotic gene receptor

The genetic mechanisms involved in Drosophila PCD are well characterized, and a key effector role is performed by the proapoptotic genes reaper (rpr), head involution defective (hid), grim and sickle (skl), collectively referred to as the RHG genes, which act by blocking the antiapoptotic regulators inhibitors of apoptosis (Bergmann et al., 1998, 2003). In line with the prominent role of RHG genes in Drosophila PCD, previous studies revealed that the RHG genes are necessary for Va PCD. To elucidate the role of dac in relation to the RHG genes, we drove the expression of rpr in dac mutants (dac, Va-Gal4 > UAS-rpr). We found a complete absence of Va neurons in this genetic background (Figure 2a–d). These results indicate that dac plays its role upstream of rpr.

3.3 dac and Abd-B do not regulate each other

The above-mentioned experiments demonstrated that dac and Abd-B are necessary for PCD in Va-PCD neurons (A5–A6).
However, only Abd-B is sufficient to trigger PCD in Va-Capa neurons (A2–A4). This raised the issue of the regulatory interplay between dac and Abd-B. To this end, we analyzed the expression of Dac in Abd-B mutants and, conversely, the expression of Abd-B in dac mutants. We did not observe any effects upon Dac or Abd-B expression in each other’s mutant background at St16 or 18hAEL (Figure 3a–c, Supplemental Figure S1A–C). These results show that Dac and Abd-B do not regulate each other during Va PCD. Moreover, we did not observe any effect on Abd-A expression in dac mutants (Figure 3d).

3.4 dac and Abd-B genetically interact with the RHG genes

To further probe the interplay between dac and Abd-B in relation to PCD, we tested their interplay with the RHG genes. Previous studies indicated that Abd-B acts via the RHG genes to control Va PCD in A5–A7 (Suska et al., 2011), and our results herein indicated that dac acts upstream of rpr to drive Va PCD. To further address the involvement of RHG genes in Va PCD and their interaction with Abd-B and dac function, we analyzed transheterozygotes between Abd-B, dac and Df(3L)H99, a genomic deletion that removes all three main RHG genes (White et al., 1994). Strikingly, while none of the double transheterozygotes displayed any ectopic survival of Va cells in A5–A6, we observed a small but significant effect in the triple transheterozygotes (Figure 4a–i). These findings support a regulatory role of Abd-B and dac in RHG genes.

3.5 Abd-B and Dac proteins can physically interact

Because both dac and Abd-B promote PCD in Va neurons and are genetically linked to the RHG genes, we postulated that Dac could act as a cofactor together with Abd-B in promoting PCD. To elucidate the possible interaction between Dac and Abd-B in vivo in Va neurons, we performed a bimolecular fluorescent complementation (BiFC) assay (Kerppola, 2008). We obtained a previously generated UAS-Abd-BVC line (Bischof et al., 2018), where the C-terminal domain of Venus had been fused to Abd-B (Hudry et al., 2011). We also generated a UAS-dacVN transgene by fusing the N-terminal domain of Venus to Dac. We expressed both fusion constructs using the dac-Gal4 or elav-Gal4 drivers. We confirmed that each transgenic construct expressed the fusion protein, as scored by Dac and Abd-B staining (Figure 5a–d″). Next, we analyzed the BiFC signal in the VNC at 18hAEL. This analysis revealed a positive BiFC signal in many neurons in the VNC (Figure 5a–d″; Supplemental Figure 2A–B). Because the Dac and Abd-B antibodies were both mouse IgG, we were not able to confirm coexpression of Abd-B and Dac together with the BiFC signal. However, we noted that the BiFC signal was present in the strongest expressing Dac or Abd-B cells (Figure 5a–d″). To probe the specificity of the Dac/Abd-B interaction, we analyzed the BiFC signal when coexpressing Abd-BVC with p53VN. This did not reveal any positive interaction (Supplemental Figure 2c). These results indicate that there is a specific physical interaction between Dac and Abd-B, apparently mostly evident in the strongest coexpressing VNC cells.
DAC MODULATES THE ACTION OF ABD-B IN VA NEURONS

To further probe the interplay between dac and Abd-B, we tested whether Abd-B misexpression, which triggers ectopic PCD in Va cells, requires dac. First, in line with previous studies (Suska et al., 2011), we found a complete absence of Va-Capa cells within the abdominal A2–A4 segments when we misexpressed Abd-B in a control background (Figure 6a,c). Next, we misexpressed Abd-B in a dac mutant background (dac; elav-Gal4 > Abd-B) and again observed complete PCD of Va cells in A2–A4, as well as in A5–A6 (Figure 6b,c). In an attempt to uncover a necessity for dac regarding the sufficiency of Abd-B to trigger PCD in Va cells (Figure 6d). These findings demonstrate that high levels of Abd-B are capable of triggering PCD in Va cells regardless of the presence or absence of dac. Thus, while dac is necessary for PCD in Va cells in A5–A6 and BiFC analysis reveal that Dac can physically interact with Abd-B, misexpression of Abd-B can overcome the need for dac activity in Va neurons.

DISCUSSION

5.1 Dac specifically supports Abd-B-mediated PCD, where Abd-B expression levels are low

Previous studies have revealed that the Hox gene Abd-B is both necessary and sufficient for PCD in posterior Va neurons (Suska et al., 2011). The PCD of Va involves the RHG motif genes rpr, grim, and hid, as well as possibly skl (Suska et al., 2011). The Abd-B expression is evident in a gradient in the VNC (Hirth et al., 1998; Karlsson et al., 2010; Miguel-Aliaga & Thor, 2004) and is very weak in A5–A6, yet it also acts to trigger PCD in Va neurons in A5–A6 (Suska et al., 2011). Here, we conducted a targeted screen to identify other genes acting to ensure PCD in Va neurons. We found that the transcriptional cofactor Dac is expressed by Va neurons in A1–A7 and acts to support Abd-B-mediated PCD of Va neurons. We do not find any evidence for cross-regulation between Abd-B and dac and instead find that they genetically interact with the Df(3L)H99 genomic deletion, which removes all three RHG genes. BiFC analysis demonstrated support for physical interaction between Abd-B and Dac.
Our results demonstrate that in a dac mutant background, the normal level of Abd-B expression is unable to trigger PCD in the A5 and A6 segments. However, overexpression of Abd-B is sufficient to execute PCD in all Va neurons (dac, elavG4 > UAS-AbdB), even in the absence of Dac. Hence, low-level Abd-B expression, as observed at the anterior limits of its expression territory, is unable to trigger PCD without Dac. By contrast, in A7, which also expresses Dac, Abd-B expression is sufficiently high to trigger PCD even without Dac. Therefore, the collaboration between Abd-B and Dac only seems to be relevant in the A5 and A6 segments. Our results strongly suggest that Dac and Abd-B physically interact to trigger A5–A6 PCD, and it is tempting to speculate that they may act directly upon the RHG genes. Future experiments should be aimed at elucidating the precise molecular mechanisms by which the binding of cofactors to Hox proteins may help support their function at the outer limits of their expression.

### 5.2 Emerging connection between Hox and RHG genes and PCD

Studies have found that Hox genes can control PCD in other postmitotic cells in the Drosophila CNS. These include dMP2/lfp7 neurons, where Abd-B acts in an anti-apoptotic manner (Miguel-Aliaga & Thor, 2004; Miguel-Aliaga et al., 2008); CCAP neurons, where Ubx and Abd-A act in an anti-apoptotic manner (Moris-Sanz et al., 2015); and subsets of motor neurons, where Ubx acts in a pro-apoptotic and Antp in an anti-apoptotic manner (Rogulja-Ortmann et al., 2008). It
is tempting to speculate that the role of Hox genes as alternatively pro- or anti-apoptotic is decided by combinatorial expression, and perhaps physical interaction, of other TFs.

In addition to their role in postmitotic cells in the CNS, Hox genes also control the PCD of subsets of neuroblasts (NBs) in Drosophila, either at the end of the embryonic or larval neurogenesis phase, where Ubx, abd-A and Abd-B act in a proapoptotic manner to remove posterior NBs by PCD (Arya et al., 2015; Bello et al., 2003; Cenci & Gould, 2005; Karlsson et al., 2010; Monedero Cobeta et al., 2017). Recent studies have revealed that Hox genes control NB PCD by directly regulating RHG genes, acting on core enhancer elements in the RHG genomic region (Bakshi et al., 2020; Ghosh et al., 2019; Khandelwal et al., 2017). Similarly, several other studies have demonstrated that Hox genes are also tightly linked to PCD in peripheral Drosophila tissues (Hueber et al., 2007; Lohmann et al., 2002; Zhai et al., 2009, 2010). These PCD-mediating roles of Hox genes again revolve around the regulation of RHG genes by acting directly on RHG enhancer elements, particularly on the rpr gene (Sorge et al., 2012; Stobe et al., 2009).

Given the expression of Hox genes in many cells within the respective Hox expression domains, their PCD-mediating activity must be tightly gated. Intriguingly, previous studies have identified several TFs cooperating with Hox genes in mediating PCD, often by acting on RHG genes (Arya et al., 2015; Bakshi et al., 2020; Ghosh et al., 2019; Khandelwal et al., 2017; Miguel-Aliaga et al., 2008; Sorge et al., 2012; Stobe et al., 2009).

Collectively, these findings indicate that Hox genes can control segment-specific diversification of the Drosophila CNS and shape peripheral tissues by (1) restricting A-P and tissue expression, (2) interacting with different, selectively expressed TFs, and (3) directly regulating RHG genes. This notion has resulted in the proposal of combinatorial TF codes for PCD that are similar to the combinatorial coding of neuronal subtype cell fate, that is, that PCD is akin to a cell fate (Miguel-Aliaga & Thor, 2009).

5.3 Hox frontier assistants

As outlined above, several TFs have been identified as cooperating with Hox proteins in triggering PCD. However, to our knowledge, Dac is the first cofactor identified that supports Hox gene function in segments where its expression level is low, and we propose the term Hox frontier assistant (HFA) as a name for this activity. It is reasonable to think that other types of factors could be modulating the frontier action of the Hox genes in other contexts and therefore contribute to the precision with which they sculpt the architecture at the outer limits of each Hox gene expression territory. To find more factors of these characteristics, it is not enough to study expression patterns in search of genes that are expressed in the limits of the Hox gene territories. In fact, the expression of Dac is not restricted to A5–A6 but rather is expressed by many interneurons and peptidergic neurons throughout the CNS (Miguel-Aliaga et al., 2004). To find factors that assist the Hox genes at their expression limits, it will be necessary to identify cells in which each Hox gene performs a monitorable function, if possible, in more than one segment and to look for genes that alter the function in outer expression limits of each Hox gene. These genes will be good candidates for HFAs.

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**CONFLICT OF INTERESTS**
The authors have declared that no competing interests exist.

**DATA AVAILABILITY STATEMENT**
All relevant data are within the paper and its Supporting Information files.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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