WILEY

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

Luis Clarembaux-Badell[1] | Pablo Baladrón-de-Juan[1] | Hugo Gabilondo[1] |
Irene Rubio-Ferrera[1] | Irene Millán[1] | Carlos Estella[3] | Félix S. Valverde-Ortega[1] |
Ignacio Monedero Cobeta[1,2] | Stefan Thor[4] | Jonathan Benito-Sipos[1]

[1]Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain

[2]Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain

[3]Departamento de Biología Molecular and Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid (CSIC-UAM), Nicolás Cabrera 1, Madrid, Spain

[4]School of Biomedical Sciences, The University of Queensland, Brisbane, Australia

Correspondence
Stefan Thor and Jonathan Benito-Sipos, Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, E 28049 Madrid, SPAIN.
Email: jonathan.benito@uam.es; s.thor@uq.edu.au

Luis Clarembaux-Badell and Pablo Baladrón-de-Juan contributed equally to this work

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 (Becker 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 detectible 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: $elav^{C155}$ = elav-Gal4 (DiAntonio et al., 2001). *Va-Gal4* (Allan et al., 2003). *UAS-dac*
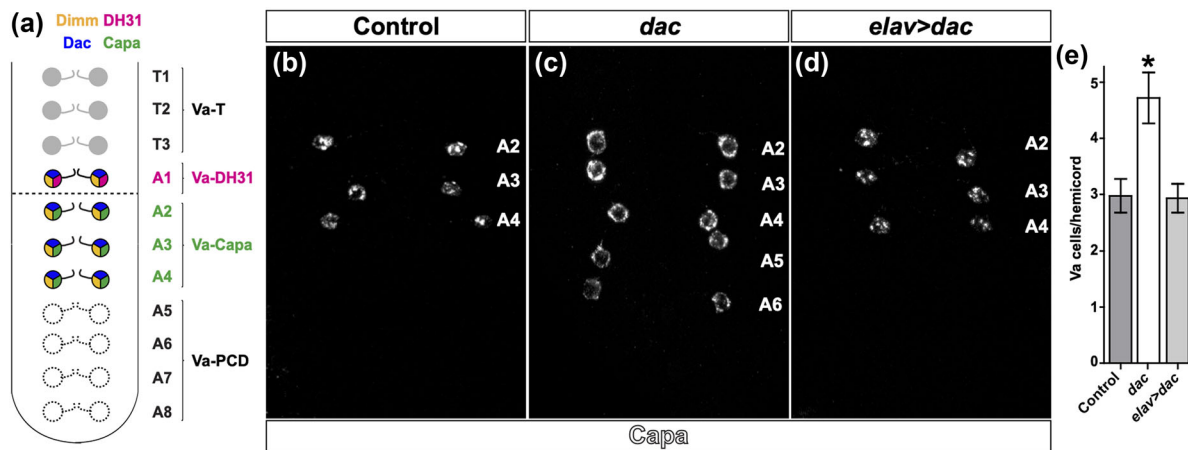
FIGURE 1 *dac* is necessary but not sufficient for PCD in Va neurons. (a) Cartoon depicting the Va cells along the Drosophila VNC and their expression of Dimm, Dac, DH31, and Capa. (b–d) Staining for Capa in the developing VNC at 18hAEL, in control, *dac³*, and *elav-Gal4/UAS-dac* embryos. (e) Quantification of Va cells per VNC hemicord at 18hAEL (asterisk denotes $p \le .05$; 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^{D18}* (obtained from E. Sanchez-Herrero). *Atu^{s1938}*, *Bgb⁹*, *bru3^{05871}*, *dac³*, *dac⁴*, *drl^{R343}*, *Dscam1^{05518}*, *dsh^{75}*, *faf^{BX4}*, *fra³*, *Fur1^{P127}*, *Hr38^{02306}*, *hth^{5E04}*, *kay¹*, *msk^{B185}*, *pan²*, *qsm^{05510}*, *rgr^{k02605}*, *rhea¹*, *rn⁵*, *run³*, *SoxN^{NC14}*, *stau^{ry9}*, *tou²*, *tsh⁸*, *wts^{x1}* (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 G**GG CGG CTC AGG CGG CAT GGA TTC TG TGA CAA GTG AAC-3′ and reverse primer 5′-CAG **TCT CGA G**GG CGG CTC AGG CGG 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 Xho1-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-Abd-B^{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
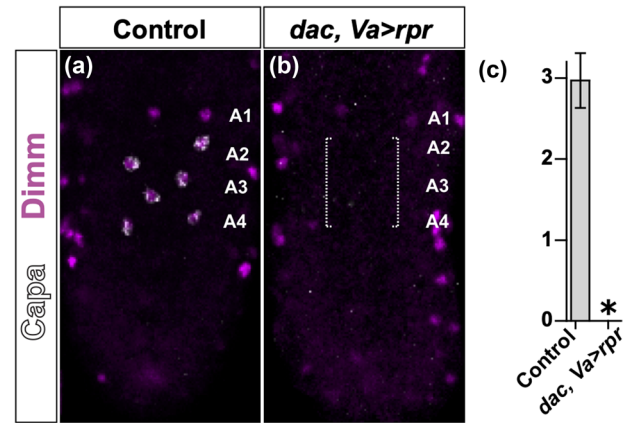
Genotype	CAPA A5–A7
Atu S1 938	–
Bgb 9	–
bru3 05871	–
Chi e5.5	–
ci 94	–
CtBP 03463	–
dac 3	+
Fur1 P127	–
Hr38 02306	–
hth 5E04	–
kay 1	–
Mfap1 DsRed	–
msk B185	–
NorpA 36	–
pan 2	–
qsm 05510	–
rgr k02605	–
rhea 1	–
rn 5	–
run 3	–
SoxN NC14	–
stau ry9	–
tou 2	–
tsh8	–
wts x1	

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 $p \leq .05$; Student's *t* test; mean ± SEM; $n > 10$ VNCs per genotype).

3 | 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).
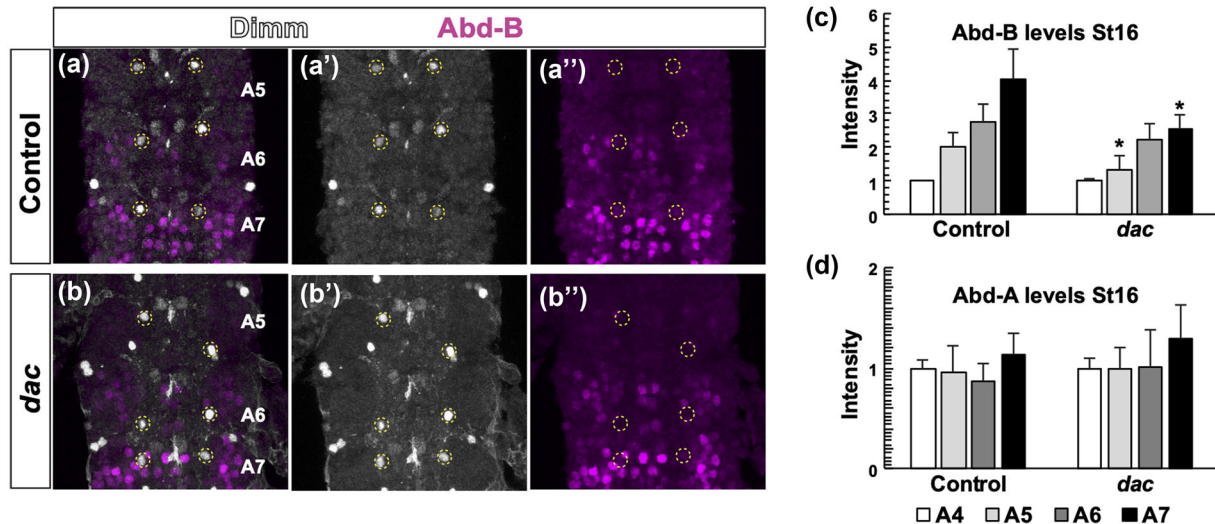
FIGURE 3 *dac* does not regulate Abd-B or Abd-A. (a,b″) Staining for Dimm and Abd-B in control and *dac* mutants at 18hAEL. (c,d) Quantification of Abd-B and Abd-A immunostaining intensity in the A4–A7 segments at 18hAEL (asterisks denote $p \leq .05$; Student's *t* test; mean ± SEM; $n > 10$ VNCs per genotype).

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 intersection 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-B^{VC}* 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-dac^{VN}* 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 5b–d″). To probe the specificity of the Dac/Abd-B interaction, we analyzed the BiFC signal when coexpressing Abd-B^{VC} with p53^{VN}. 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.
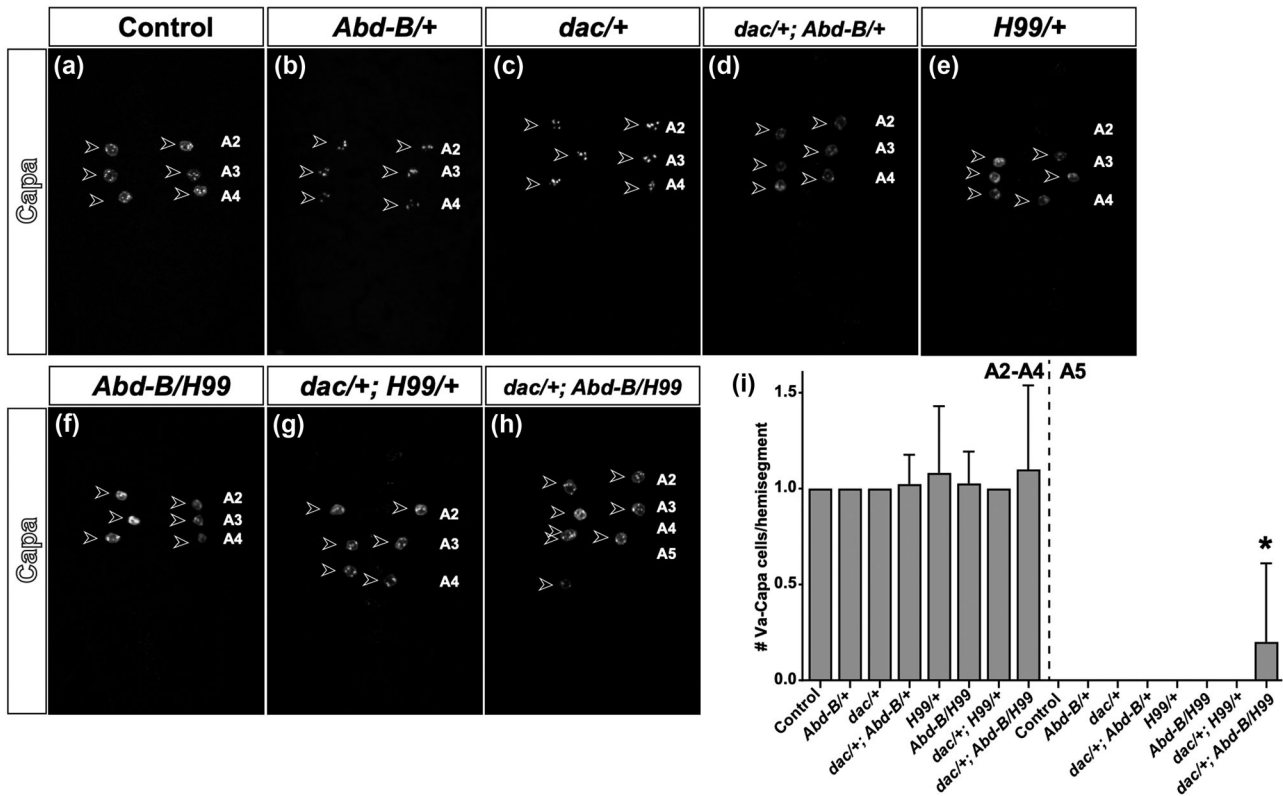
FIGURE 4 *dac*, *Abd-B*, and *H99* genetically interact to trigger PCD. (A–H) Staining for Capa in the developing VNC at 18hAEL, in control, *dac³*, *Abd-B* and *Df(3L)H99*, single, double, and triple transheterozygotic embryos. (i) Quantification of Va cells per A2–A4 or A5 VNC hemisegments, at 18hAEL (asterisk denotes $p \leq .05$; Student's *t* test; mean ± SD; $n \geq 6$ VNCs per genotype)

# 4 | 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* in the *Abd-B* misexpression experiments, we repeated the experiment at +17˚C because the Gal4/UAS system is known to express less at lower temperatures (Duffy, 2002). However, this still failed to reveal any 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.

5 | 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.
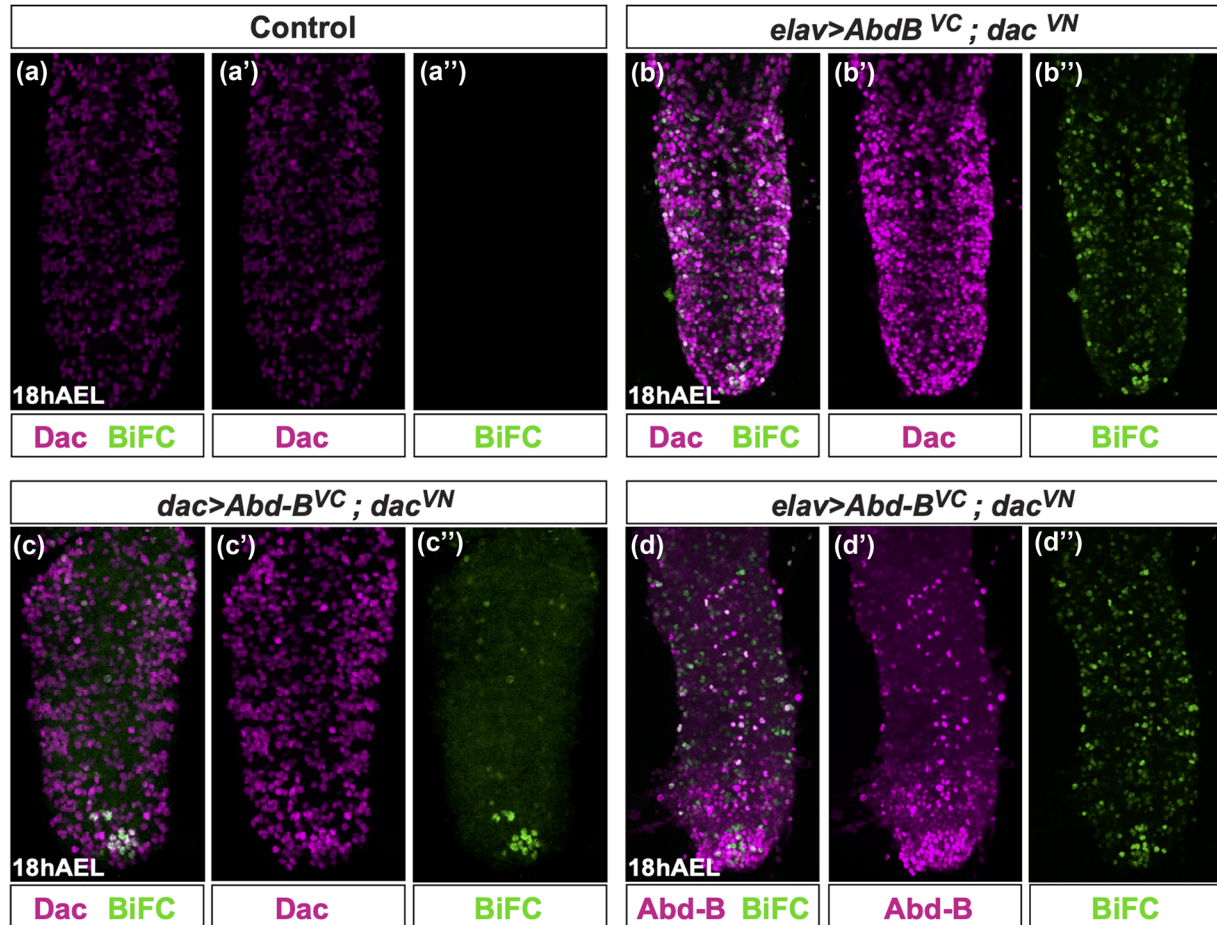
FIGURE 5 BiFC reveals that Dac and Abd-B physically interact. (a–d″) BiFC and staining for Dac and Abd-B in *dac-Gal4* or *elav-Gal4* crossed to *UAS-Abd-B^{VC}* and *UAS-dac^{VN}* transgenic embryos at 18hAEL

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 Dac to Abd-B facilitates the PCD process and why high levels of Abd-B do not require Dac.

Studies of Va neurons provide evidence for how Hox genes can control neuronal subtype specification with different combinations of other Transcription Factors (TFs) and in the case of Va neurons: *Antp* specifies Va-X, likely acting with an unidentified factor(s); *Ubx* specifies Va-DH31, acting with *dimm*; *abd-A* specifies Va-Capa, acting with *dimm*; and *Abd-B* specifies Va-PCD, acting with *dac* [herein; (Gabilondo et al., 2011, 2018; Suska et al., 2011)]. The Dac/Abd-B physical interaction may indicate that 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/Ilp7 neurons, where *Abd-B* acts in an antiapoptotic manner (Miguel-Aliaga & Thor, 2004; Miguel-Aliaga et al., 2008); CCAP neurons, where *Ubx* and *Abd-A* act in an antiapoptotic 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
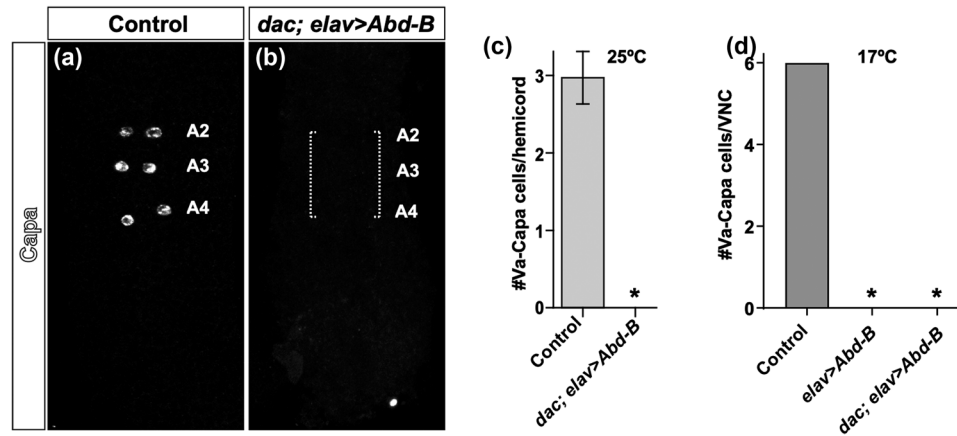
FIGURE 6 *Abd-B* is sufficient for PCD in *dac* mutants. (a,b) Staining for Capa in the developing VNC at 18hAEL, in control and *dac³*; *elav-Gal4/UAS-Abd-B* embryos. (c,d) Quantification of Va cells per VNC hemicord at 18hAEL, at +25°C or +17°C (asterisks denote $p \leq .05$; Student's *t* test; mean ± SD; $n \geq 8$ VNCs per genotype).

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.

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.

ORCID

Jonathan Benito-Sipos https://orcid.org/0000-0002-1159-3644

REFERENCES

Allan, D. W., Park, D., St Pierre, S. E., Taghert, P. H., & Thor, S. (2005). Regulators acting in combinatorial codes also act independently in single differentiating neurons. *Neuron*, *45*, 689–700.

Allan, D. W., Pierre, S. E., Miguel-Aliaga, I., & Thor, S. (2003). Specification of neuropeptide cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell*, *113*, 73–86.

Arya, R., Sarkissian, T., Tan, Y., & White, K. (2015). Neural stem cell progeny regulate stem cell death in a Notch- and Hox-dependent manner. *Cell Death and Differentiation*, *22*, 1378–1387.

Arya, R., & White, K. (2015). Cell death in development: Signaling pathways and core mechanisms. *Seminars in Cell & Developmental Biology*, *39*, 12–19.

Bakshi, A., Sipani, R., Ghosh, N., & Joshi, R. (2020). Sequential activation of Notch and Grainyhead gives apoptotic competence to Abdominal-B-expressing larval neuroblasts in the Drosophila central nervous system. *PLoS Genetics*, *16*, e1008976.

Baumgardt, M., Miguel-Aliaga, I., Karlsson, D., Ekman, H., & Thor, S. (2007). Specification of neuronal identities by feedforward combinatorial coding. *PLoS Biology*, *5*, 295–308.

Becker, H., Renner, S., Technau, G. M., & Berger, C. (2016). Cell-Autonomous and Non-cell-autonomous function of Hox genes specify segmental neuroblast identity in the gnathal region of the embryonic CNS in Drosophila. *PLoS Genetics*, *12*, e1005961.

Bello, B. C., Hirth, F., & Gould, A. P. (2003). A pulse of the Drosophila Hox protein Abdominal-A schedules the end of neural proliferation via neuroblast apoptosis. *Neuron*, *37*, 209–219.

Bergmann, A., Agapite, J., & Steller, H. (1998). Mechanisms and control of programmed cell death in invertebrates. *Oncogene*, *17*, 3215–3223.

Bergmann, A., Yang, A. Y., & Srivastava, M. (2003). Regulators of IAP function: Coming to grips with the grim reaper. *Current Opinion in Cell Biology*, *15*, 717–724.

Birkholz, O., Rickert, C., Berger, C., Urbach, R., & Technau, G. M. (2013). Neuroblast pattern and identity in the Drosophila tail region and role of doublesex in the survival of sex-specific precursors. *Development (Cambridge, England)*, *140*, 1830–1842.

Bischof, J., Duffraisse, M., Furger, E., Ajuria, L., Giraud, G., Vanderperre, S., Paul, R., Bjorklund, M., Ahr, D., Ahmed, A. W., Spinelli, L., Brun, C., Basler, K., & Merabet, S. (2018). Generation of a versatile BiFC ORFeome library for analyzing protein-protein interactions in live Drosophila. *Elife*, *7*, e38853.

Bischof, J., Maeda, R. K., Hediger, M., Karch, F., & Basler, K. (2007). Optimized transgenesis system for Drosophila using germline-specific phiC31 integrases. *PNAS*, *104*, 3312–3317.

Cenci, C., & Gould, A. P. (2005). Drosophila Grainyhead specifies late programmes of neural proliferation by regulating the mitotic activity and Hox-dependent apoptosis of neuroblasts. *Development (Cambridge, England)*, *132*, 3835–3845.

DiAntonio, A., Haghighi, A. P., Portman, S. L., Lee, J. D., Amaranto, A. M., & Goodman, C. S. (2001). Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature*, *412*, 449–452.

Duffy, J. B. (2002). GAL4 system in Drosophila: A fly geneticist's Swiss army knife. *Genesis (New York, N.Y.: 2000)*, *34*, 1–15.

Gabilondo, H., Losada-Perez, M., del Saz, D., Molina, I., Leon, Y., Canal, I., Torroja, L., & Benito-Sipos, J. (2011). A targeted genetic screen identifies crucial players in the specification of the Drosophila abdominal Capaergic neurons. *Mechanisms of Development*, *128*, 208–221.

Gabilondo, H., Rubio-Ferrera, I., Losada-Perez, M., Del Saz, D., Leon, Y., Molina, I., Torroja, L., D, W. A., & Benito-Sipos, J. (2018). Segmentally homologous neurons acquire two different terminal neuropeptidergic fates in the Drosophila nervous system. *PLoS One*, *13*, e0194281.

Ghosh, N., Bakshi, A., Khandelwal, R., Rajan, S. G., & Joshi, R. (2019). The Hox gene Abdominal-B uses doublesex(F) as a cofactor to promote neuroblast apoptosis in the Drosophila central nervous system. *Development (Cambridge, England)*, *146*, .

Hewes, R. S., Park, D., Gauthier, S. A., Schaefer, A. M., & Taghert, P. H. (2003). The bHLH protein Dimmed controls neuroendocrine cell differentiation in Drosophila. *Development (Cambridge, England)*, *130*, 1771–1781.

Hirth, F., Hartmann, B., & Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development (Cambridge, England)*, *125*, 1579–1589.

Hudry, B., Viala, S., Graba, Y., & Merabet, S. (2011). Visualization of protein interactions in living Drosophila embryos by the bimolecular fluorescence complementation assay. *BMC Biology*, *9*, 5.

Hueber, S. D., Bezdan, D., Henz, S. R., Blank, M., Wu, H., & Lohmann, I. (2007). Comparative analysis of Hox downstream genes in Drosophila. *Development (Cambridge, England)*, *134*, 381–392.

Karlsson, D., Baumgardt, M., & Thor, S. (2010). Segment-specific neuronal subtype specification by the integration of anteroposterior and temporal cues. *PLoS Biology*, *8*, e1000368.

Kerppola, T. K. (2008). Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annual Review of Biophysics*, *37*, 465–487.

Khandelwal, R., Sipani, R., Govinda Rajan, S., Kumar, R., & Joshi, R. (2017). Combinatorial action of Grainyhead, extradenticle and notch in regulating Hox mediated apoptosis in Drosophila larval CNS. *PLoS Genetics*, *13*, e1007043.

Lohmann, I., McGinnis, N., Bodmer, M., & McGinnis, W. (2002). The Drosophila Hox gene deformed sculpts head morphology via direct regulation of the apoptosis activator reaper. *Cell*, *110*, 457–466.

Merabet, S., Litim-Mecheri, I., Karlsson, D., Dixit, R., Saadaoui, M., Monier, B., Brun, C., Thor, S., Vijayraghavan, K., Perrin, L., & Pradel, J. y G. Y. (2011). Insights into Hox protein function from a large-scale combinatorial analysis of protein domains. *PLoS Genetics*, *7*(10), e1002302. https://doi.org/10.1371/journal.pgen.1002302 [doi]

Miguel-Aliaga, I., Allan, D. W., & Thor, S. (2004). Independent roles of the dachshund and eyes absent genes in BMP signaling, axon

pathfinding and neuronal specification. *Development (Cambridge, England)*, 131, 5837–5848.

Miguel-Aliaga, I., & Thor, S. (2004). Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. *Development (Cambridge, England)*, 131, 6093–6105.

Miguel-Aliaga, I., & Thor, S. (2009). Programmed cell death in the nervous system—a programmed cell fate? *Current Opinion in Neurobiology*, 19, 127–133.

Miguel-Aliaga, I., Thor, S., & Gould, A. P. (2008). Postmitotic specification of Drosophila insulinergic neurons from pioneer neurons. *PLoS Biology*, 6, e58.

Monedero Cobeta, I., Salmani, B. Y., & Thor, S. (2017). Anterior-posterior gradient in neural stem and daughter cell proliferation governed by spatial and temporal Hox control. *Current Biology*, 27, 1161–1172.

Moris-Sanz, M., Estacio-Gomez, A., Sanchez-Herrero, E., & Diaz-Benjumea, F. J. (2015). The study of the Bithorax-complex genes in patterning CCAP neurons reveals a temporal control of neuronal differentiation by Abd-B. *Biology Open*, 4, 1132–1142.

Park, D., Hadzic, T., Yin, P., Rusch, J., Abruzzi, K., Rosbash, M., Skeath, J. B., Panda, S., Sweedler, J. V., & Taghert, P. H. (2011). Molecular organization of Drosophila neuroendocrine cells by Dimmed. *Current Biology*, 21, 1515–1524.

Rogulja-Ortmann, A., Renner, S., & Technau, G. M. (2008). Antagonistic roles for Ultrabithorax and Antennapedia in regulating segment-specific apoptosis of differentiated motoneurons in the Drosophila embryonic central nervous system. *Development (Cambridge, England)*, 135, 3435–3445.

Rogulja-Ortmann, A., & Technau, G. M. (2008). Multiple roles for Hox genes in segment-specific shaping of CNS lineages. *Fly (Austin)*, 2, 316–319.

Schmid, A., Chiba, A., & Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: Neural cell types, axon projections and muscle targets. *Development (Cambridge, England)*, 126, 4653–4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J., & Technau, G. M. (1997). The embryonic central nervous system lineages of Drosophila melanogaster. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Developmental Biology*, 189, 186–204.

Shen, W., & Mardon, G. (1997). Ectopic eye development in Drosophila induced by directed dachshund expression. *Development (Cambridge, England)*, 124, 45–52.

Sorge, S., Ha, N., Polychronidou, M., Friedrich, J., Bezdan, D., Kaspar, P., Schaefer, M. H., Ossowski, S., Henz, S. R., Mundorf, J., Rätzer, J., Papagiannouli, F., & Lohmann, I. (2012). The cis-regulatory code of Hox function in Drosophila. *Embo Journal*, 31, 3323–3333.

Stobe, P., Stein, M. A., Habring-Muller, A., Bezdan, D., Fuchs, A. L., Hueber, S. D., Wu, H., & Lohmann, I. (2009). Multifactorial regulation of a hox target gene. *PLoS Genetics*, 5, e1000412.

Stratmann, J., Ekman, H., & Thor, S. (2019). A branching gene regulatory network dictating different aspects of a neuronal cell identity. *Development (Cambridge, England)*, 146, dev174300.

Stratmann, J., & Thor, S. (2017). Neuronal cell fate specification by the molecular convergence of different spatio-temporal cues on a common initiator terminal selector gene. *PLoS Genetics*, 13, e1006729.

Suska, A., Miguel-Aliaga, I., & Thor, S. (2011). Segment-specific generation of Drosophila Capability neuropeptide neurons by multi-faceted Hox cues *Developmental Biology*, 353, 72–80.

Urbach, R., & Technau, G. M. (2004). Neuroblast formation and patterning during early brain development in Drosophila. *Bioessays*, 26, 739–751.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., & Steller, H. (1994). Genetic control of programmed cell death in Drosophila. *Science*, 264, 677–683.

Yaghmaeian Salmani, B., Monedero Cobeta, I., Rakar, J., Bauer, S., Curt, J. R., Starkenberg, A., & Thor, S. (2018). Evolutionarily conserved anterior expansion of the central nervous system promoted by a common PcG-Hox program. *Development (Cambridge, England)*, 145, .

Zhai, Z., Fuchs, A. L., & Lohmann, I. (2010). Cellular analysis of newly identified Hox downstream genes in Drosophila. *European Journal of Cell Biology*, 89, 273–278.

Zhai, Z., Stein, M. A., & Lohmann, I. (2009). Expression of the apoptosis gene reaper in homeotic, segmentation and other mutants in Drosophila. *Gene Expression Patterns*, 9, 357–363.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L. M., Steller, H., & Nambu, J. R. (1997). Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. *PNAS*, 94, 5131–5136.

SUPPORTING INFORMATION

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