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The *Drosophila* homeodomain transcription factor Homeobrain is involved in the formation of the embryonic protocerebrum and the supraesophageal brain commissure

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ABSTRACT

During the embryonic development of *Drosophila melanogaster* many transcriptional activators are involved in the formation of the embryonic brain. In our study we show that the transcription factor Homeobrain (Hbn), a member of the 57B homeobox gene cluster, is an additional factor involved in the formation of the embryonic *Drosophila* brain. Using a Hbn antibody and specific cell type markers a detailed expression analysis during embryonic brain development was conducted. We show that Hbn is expressed in several regions in the protocerebrum, including fibre tract founder cells closely associated with the supraesophageal brain commissure and also in the mushroom bodies. During the formation of the supraesophageal commissure, Hbn and FasII-positive founder cells build an interhemispheric bridge priming the commissure and thereby linking both brain hemispheres. The Hbn expression is restricted to neural but not glial cells in the embryonic brain. In a mutagenesis screen we generated two mutant *hbn* alleles that both show embryonic lethality. The phenotype of the *hbn* mutant alleles is characterized by a reduction of the protocerebrum, a loss of the supraesophageal commissure and mushroom body progenitors and also by a dislocation of the optic lobes. Extensive apoptosis correlates with the impaired formation of the embryonic protocerebrum and the supraesophageal commissure. Our results show that Hbn is another important factor for embryonic brain development in *Drosophila melanogaster*.

1. Introduction

The embryonic *Drosophila* brain consists of the supraesophageal and the subesophageal ganglia. These major parts are subdivided in three compartments each, the so called neuromeres, including protocerebrum (b1), deutocerebrum (b2) and tritocerebrum (b3) for the supraesophageal part and mandibular, maxillary and labial neuromeres for the subesophageal part of the brain (Younossi-Hartenstein et al., 1996; for review see Reichert and Boyan, 1997). The neuromeres of the supraesophageal ganglia and the segments of the ventral nerve cord are each connected by a commissure. The protocerebral part of the two hemispheres is linked by the supraesophageal brain commissure, the deutocerebrum by the frontal commissure and the tritocerebrum by the tritocerebral commissure (Nassif et al., 1998).

Subareas of the neuroectoderm, which are specified to become the procephalic neurogenic region through genetic interactions during gastrulation, give rise to the anterior part of the brain (Jürgens and Hartenstein, 1993). The posterior brain derives from the most rostrally located ventral neurogenic region (reviewed in Doe and Skeath, 1996). These events of embryonic brain development start in embryonic stage 8 with the delamination of neuronal precursor cells, called neuroblasts (Campos-Ortega and Hartenstein, 1997), in the procephalic neuroectoderm. This process is regulated by the proneural genes of the Achaete-Scute-Complex (AS-C) (see Campos-Ortega, 1995 for review), which are necessary to generate a competence group of cells with the potential to become neuroblasts. The subsequent selection of the neuroblast through lateral inhibition is regulated by the Delta/Notch pathway (Muskavitch, 1994). The results of this early neurogenesis are 108 bilaterally arranged neuroblasts that are generated until embryonic stage 12 (Urbach and Technau, 2003). Each neuroblast divides asymmetrically and thereby generates through self-renewal a further neuroblast and a neuronal precursor cell, the ganglion mother cell (GMC),

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which will then divide symmetrically and produce two neurons. In this way the neuroblast produces embryonic lineages of primary neurons (reviewed in Doe, 2008). This mode of division is typical for type I neuroblasts that make up most of the cell lineages in the embryonic brain. In contrast to type I neuroblasts, the solely existing 8 type II neuroblasts generate intermediate neural progenitor cells (INPs) that divide several times to generate GMCs, which in turn divide into two neurons (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). The type II neuroblasts that generate larger lineages during larval brain development are already present in later stages of embryonic brain development (Walsh and Doe, 2017; Álvarez and Díaz-Benjumea, 2018). At the end of embryogenesis, most neuroblasts undergo a period of quiescence and resume their division during the larval stage. The only type I neuroblasts in the embryo that do not enter quiescence are the four mushroom body neuroblasts (MBNBs) that generate 30–40 cells in the embryo (Kunz et al., 2012) and give rise to the mushroom bodies throughout larval development which function as centres for olfactory learning and memory (for reviews see Heisenberg, 2003 and Davis, 2005).

When all the neuroblasts in the embryonic brain have been selected and their corresponding lineages have established a scaffold of longitudinal tracts, several brain commissures are formed (Therianos et al., 1995). The most prominent commissure is the supraesophageal commissure connecting both brain hemispheres in the protocerebrum. This commissure is pioneered by axons projecting from both hemispheres towards the midline in close association with an interhemispheric bridge (Therianos et al., 1995; Boyan et al., 2003). These axons derive from fibre tract founder cells including P2l and P2m, which pioneer the supraesophageal commissure and build an axon bundle called the anterior ventral commissural tract (VCT) (Nassif et al., 1998; Younossi-Hartenstein et al., 2006). Later, the founder cells P3l and P4l form the dorsal commissural tract (DCT) to complete formation of the commissure.

Several transcription factors are involved in the genetic regulation of embryonic brain development. The first identified factors were the head gap genes *tailless* (*tll*) (Strecker et al., 1986), *orthodenticle* (*otd*) (Finkelstein et al., 1990), *empty spiracles* (*ems*) (Dalton et al., 1989; Walldorf and Gehring, 1992) and *buttonhead* (*btd*) (Wimmer et al., 1993) which show overlapping expression domains in the head neuroectoderm. The most dramatic brain phenotype is visible in *tll* mutants, which show complete loss of the protocerebrum including the supraesophageal commissure (Strecker et al., 1986), whereas in *otd* mutants a large part of the protocerebrum and a smaller part of the deutocerebrum is missing (Hirth et al., 1995). *Ems* and *btd* have overlapping effects in the deutocerebrum and tritocerebrum (Hirth et al., 1995; Younossi-Hartenstein et al., 1997). The common function of all head gap genes is that they are necessary for the expression of the proneural gene *lethal of scute* (*l'sc*) within their specific expression domains. If *l'sc* is not activated properly in this location, no neuroblasts will be generated and the corresponding parts of the brain will be missing (Younossi-Hartenstein et al., 1997).

The most prominent class of transcription factors expressed in the brain is encoded by the homeobox genes. Examples are not only the gap genes *otd* and *ems*, but also *unplugged* (*upg*) (Chiang et al., 1995), *muscle segment homeobox* (*msh*) (Isshiki et al., 1997), *ventral nervous system defective* (*vnd*) (McDonald et al., 1998) and others. They are acting along the anterior-posterior as well as dorsal-ventral axis in neuroblasts (Urbach and Technau, 2003) and primary neuronal clusters (Sprecher et al., 2007) and highly conserved during development. Many of their mammalian homologs called *Hox* genes are also required for brain development, a good example is *otd* and its mammalian homologs *Otx1/2* (Simeone, 1998) which can even functionally replace each other in rescue experiments (Nagao et al., 1998; Leuzinger et al., 1998; Acampora et al., 1998). Three other homeobox genes expressed in the brain are located close to each other in a homeobox gene cluster in the 57B region of the second chromosome. These genes are *orthopedia* (*otp*) (Simeone et al., 1994; Hildebrandt et al., 2020), *Drosophila retinal homeobox* (*DRx*) (Mathers et al., 1997; Eggert et al., 1998) and *homeobrain* (*hbn*) (Walldorf et al., 2000). For these genes it was recently shown that they belong to a group of eight genes which are important for brain progenitor cell generation and proliferation thereby driving the anterior expansion of the CNS (Curt et al., 2019). They are expressed in embryonic type II neuroblast and are important for the generation of their offspring. Also wing disc cells can be reprogrammed by these factors into brain neural progenitor cells (Curt et al., 2019).

In this paper we focus on one of these important factors, Hbn, and show its detailed expression pattern throughout embryonic brain development. This analysis shows that Hbn is expressed mainly in protocerebral lineages of dorsomedial and ventral subareas of the protocerebrum. A cell type-specific analysis showed that this expression occurs in neuroblasts, GMCs and neurons. The function of Hbn during the formation of the supraesophageal commissure starts with its expression in commissure founder cells during earlier development of the brain, which stay in close association with the formed interhemispheric bridge and are thus necessary for the generation of additional axonal tracts that finally generate the complete commissure. For a functional analysis of *hbn* we isolated two EMS-induced embryonic lethal mutant alleles of *hbn*. Their phenotypes are characterized by severe defects in the protocerebrum where the supraesophageal commissure and parts of the brain including mushroom body progenitors are missing. These features are caused by massive apoptosis of cells in the brain. These findings reveal an important function of Hbn during embryonic brain development of *Drosophila*.

2. Results

2.1. Embryonic expression pattern of Hbn

For a detailed analysis of the Hbn expression pattern at the cellular level, we generated an anti-Hbn antibody and first stained different embryonic stages of wild-type embryos focusing on the head region. In the early embryonic stage 4, broad anterior blastodermal expression of Hbn was detectable (Fig. 1A) which changed through anterior and ventral repression to a horseshoe-like stripe in dorsal and lateral regions in embryonic stage 5 (Fig. 1B). In embryonic stage 6, this stripe was altered to exhibit a V-shaped pattern with two lateral domains of intensive antibody staining (Fig. 1C, blue arrowhead), a central domain of weaker staining (Fig. 1C, pink arrowhead) and transition zones between the central and lateral domains with even weaker staining. The expression pattern expanded in embryonic stage 7, and two additional zones of Hbn expression emerged close to the lateral zones (Fig. 1D, orange arrowhead, compare to Fig. 1C). In addition, the V-shaped pattern stretched posteriorly, and cells in the midline between the lateral domains and the central domain were also stained. In stage 8, complex morphogenetic movements occurred in the embryo (Robertson et al., 2003), and the first neuroblast delaminations were initiated in the procephalic neuroectoderm (Urbach and Technau, 2003). During this stage, Hbn expression was visible in two additional lateral regions near the head furrow (Fig. 1E, white arrowhead), whereas in the medial part of the V-shaped expression domain, Hbn expression decreased (Fig. 1E, pink arrowhead). Simultaneously, the expression intensity in the lateral parts remained more or less constant, but a second new expression domain was present at the anterior part of the embryo in the clypeolabral region (Fig. 1E, green arrowhead). At this timepoint, all the expression domains appeared more or less connected. In stage 9, the medial expression was almost gone, and the expression domains were more separated from each other (Fig. 1F, white, orange and blue arrowheads) and from the clypeolabral expression spot (Fig. 1F, green arrowhead). When the two brain hemispheres started to form in embryonic stage 10, the posterior expression domains were more separated (Fig. 1G, white arrowhead), and the clypeolabral expression of Hbn was broader (Fig. 1G, green arrowhead). In stage 12, the Hbn expression pattern started to concentrate in the growing hemispheres of the young

brain. At the posterior part of the hemispheres some Hbn- expressing cells orientated to the midline occur and formed a kind of protrusion (Fig. 1H, yellow arrowhead) whereas the clypeolabral expression was decreasing (Fig. 1H, green arrowhead). During further development in embryonic stage 13 and 14, the Hbn expressing cells at the anterior part of the hemispheres in the protocerebrum increased, and the posterior cells formed an interhemispheric bridge of Hbn-positive cells (Fig. 1I & J, yellow arrowhead). The clypeolabral Hbn expression disappeared in stage 14, and a few Hbn- positive cells could be detected (Fig. 1J, purple arrowhead) in the tritocerebrum (see also Supplementary Fig. 1 for assigning the Hbn expression to different neuromeres). In stage 15, a strong Hbn signal was detected in both brain hemispheres, mainly in the protocerebrum, in the deutocerebrum and positive cells in the tritocerebrum. In this stage, a retraction of the Hbn-expressing bridge cells was visible (Fig. 1K, yellow arrowhead). In stage 17, the Hbn expression pattern was strongly reduced in all areas, especially in the protocerebrum.

To assign the Hbn expression to specific brain regions we then performed staining at stage 15, when the brain and inter hemispheric bridge expression of Hbn was clearly visible, in combination with HRP, a general neuronal marker for sensory neurons, peripheral nerves and all fibre tracts (Jan and Jan, 1982). Nine optical sections of the brain from

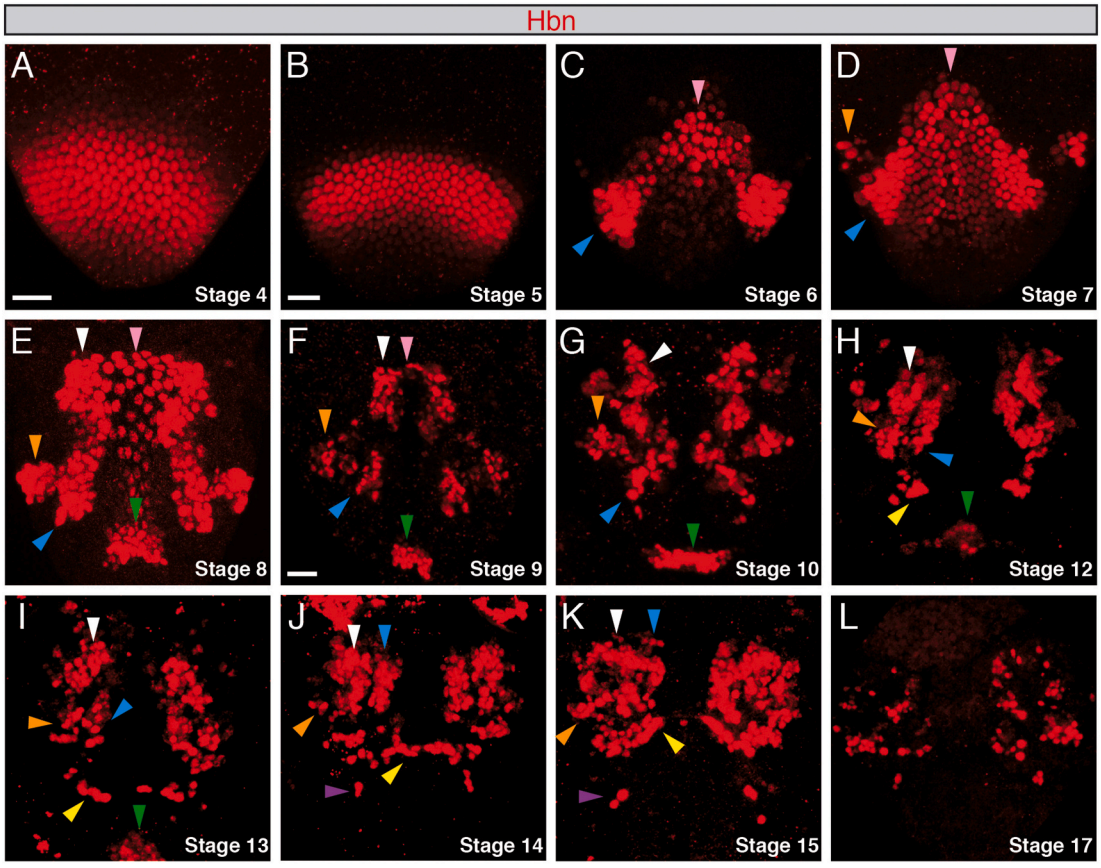

Fig. 1. Homeobrain expression during *Drosophila* embryonic development.
Laser confocal images of the anterior part of *Drosophila* embryos labeled with an anti-Hbn antibody (red). All views are from the dorsal side, the anterior ends of the embryos are pointing to the bottom. Stages were determined according to Campos-Ortega and Hartenstein (1997) and are indicated in the figure. For symmetrical expression domains arrowheads are only shown for the left side.
(A) Broad anterior blastoderm expression of Hbn.
(B) Dorsal anterior stripe of Hbn-expressing blastodermal cells.
(C) Splitting of the anterior stripe into two lateral domains (blue arrowhead) and a central domain (pink arrowhead), so the pattern now appears V-shaped.
(D) The V-shaped pattern expands (pink arrowhead), and two additional small lateral domains arise (orange arrowhead). Faintly stained cells are visible between the lateral domains and the central domain.
(E) The Hbn expression pattern consists of a multi domain pattern with an extended central region (white and pink arrowheads) and lateral zones (blue and orange arrowheads). In the anterior region a new expression domain in the clypeolabrum is visible (green arrowhead).
(F) A procephalic pattern consisting of a horseshoe-shaped-pattern (white and pink arrowheads), the two lateral zones (blue and orange arrowheads) and the clypeolabral domain (green arrowhead) is visible. The midline expression is lost.
(G) The clypeolabral Hbn expression is maintained (green arrowhead), and the other expression domains remain separated (blue, orange and white arrowheads).
(H) Hbn expression is maintained in discrete regions, albeit more condensed, with the formation of cells building a kind of protrusion towards the midline is visible (yellow arrowhead).
(I) The midline between both hemispheres shows Hbn-positive cells (yellow arrowhead).
(J) An interhemispheric bridge of Hbn-expressing cells (yellow arrowhead) is detectable at the midline. The Hbn expression is divided into a medial (blue arrowhead), anterior central (white arrowhead) and a lateral domain (orange arrowhead). A few Hbn-expressing cells emerge in the tritocerebrum (purple arrowhead).
(K) The midline crossing bridge of Hbn-expressing cells is withdrawn (yellow arrowhead), and the Hbn expression in the other domains is similar to stage 14 but appears more compact (blue, orange, yellow and white arrowheads).
(L) In stage 17 embryos the expression pattern is strongly reduced.
(Scale bars: 20 μm; G–L like A, C–E like B.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the ventral to the dorsal side according to neuraxis are shown in Fig. 2. In ventrally located sections (Fig. 2A–C), Hbn expression was visible in ventromedial (VM) and ventrolateral (VL) regions, in a deeper section also in a ventrocentral (VC) region. Cells from the prominent dorsomedial (DM) region were also visible in this location. In the middle between the ventral and dorsal sections (Fig. 2D–F), where the supraesophageal commissure (SEC) is the most prominent structure, Hbn expression was still visible dorsomedial (DM) and in the region of the mushroom bodies (MB). Hbn-expressing cells were also in close association with the supraesophageal commissure (white arrowheads), laterally on both sides of the commissure (yellow arrowheads) and around the protocerebral connectives (PCN). In more dorsal sections (Fig. 2G–H), the expression was less pronounced with a few cells in both brain hemispheres, including some cells in a dorsocentral (DC) position, close to the optic lobe (OL) and near the tritocerebral commissure (TC) (white arrowheads).

The dynamics of the detected Hbn expression pattern could be a result of spatial and temporal regulation of expression and of the proliferation and rearrangement of different tissues, but suggested a contribution of Hbn in the development of the embryonic brain and probably of the brain commissure connecting both brain hemispheres.

2.2. *Hbn is expressed in neuroblasts, GMCs and neurons, but not in glial cells*

For a precise characterization of the neuronal identity of Hbn a colocalization study with several specific neuronal markers was carried out to determine their cellular identities in the brain. As general neuronal markers we used Nrt (Barthalay et al., 1990) to visualize all primary neurons in the embryo and HRP. To analyse Hbn expression in neuroblasts (NBs), Deadpan (Dpn) was used as a specific marker for nervous system stem cells (Bier et al., 1992). Colocalization of Hbn and Dpn was found in several cells close to the midline and in the outermost lateral ones, as well as close to the head furrow below the procephalic neuroectoderm at stage 10 (Fig. 3A, arrowheads). Altogether, Hbn expression could be detected in approximately 13 cells per brain hemisphere, most likely neuroblasts. In the next step, we performed a simultaneous staining with anti-Prospero and anti-Elav antibodies to investigate Hbn expression in ganglion mother cells (GMCs), which arise by asymmetric divisions of neuroblasts. Prospero expression is observed in the cytoplasm in neuroblasts and in the nucleus in GMCs as well as in postmitotic neurons (Ikeshima-Kataoka et al., 1997; Berger et al., 2007). In contrast, Elav expression is only detectable in postmitotic neurons (Campos et al., 1987; Robinow and White, 1991). An Elav-negative cell with Prospero nuclear staining indicates a GMC identity. We detected several Hbn and Prospero-positive and Elav-negative cells in the dorsal anterior part of the protocerebrum at stage 12 (Fig. 3B, arrowheads) verifying the expression of Hbn in GMCs. To distinguish Hbn expression between GMCs and neurons, we again used Elav as a marker for neurons and found at the same stage a broad distribution of Hbn and Elav-positive cells throughout the brain, which, as expected more concentrated in the centre of the brain (Fig. 3C, arrowheads). To examine whether Hbn was also expressed in glial cells, we used Repo as a general glial cell marker (Campbell et al., 1994; Halter et al., 1995). In a colocalization experiment with anti-Repo and anti-Hbn antibodies, no colocalization of the proteins was detectable during embryonic

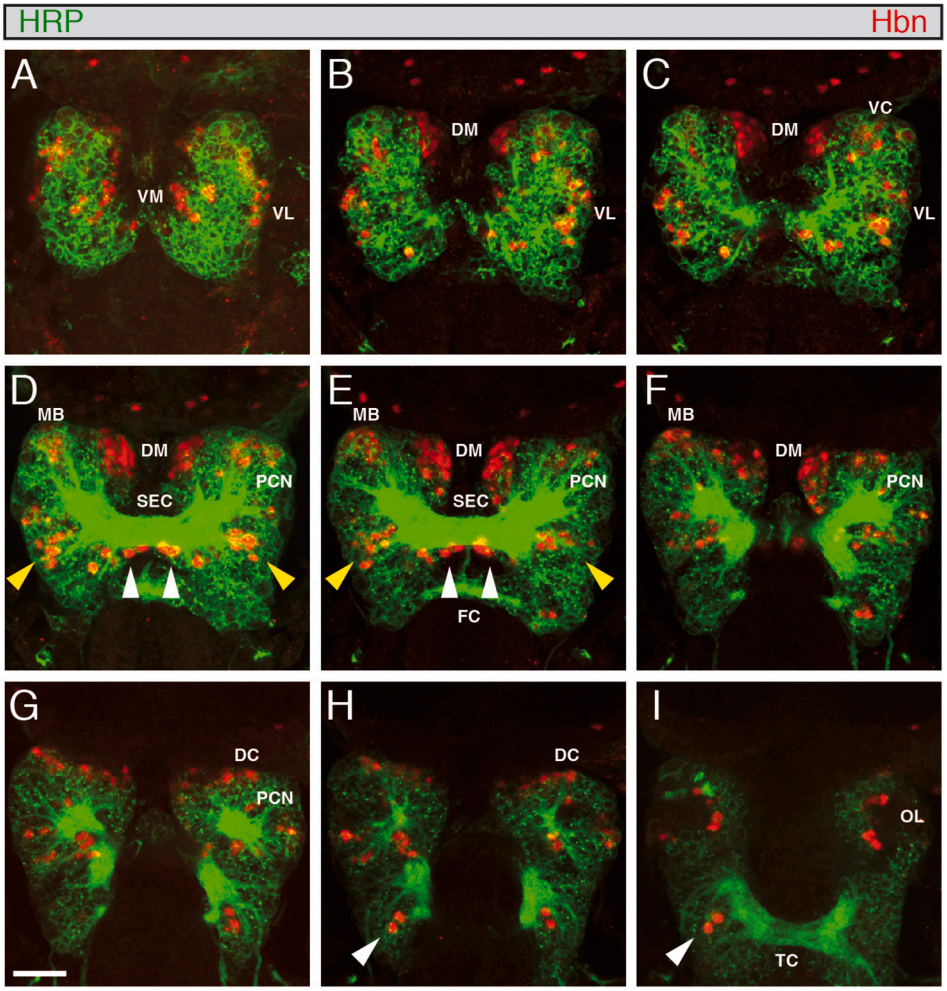

Fig. 2. Expression of Homeobrain in a stage 15 embryonic brain.
Sections of an embryonic brain at stage 15 from the ventral to the dorsal side according to neuraxis. Hbn expression is shown in red and HRP expression in green. Abbreviations are: DM dorsomedial; DC, dorsocentral; FC, frontal commissure; MB, mushroom bodies; OL, optic lobe; PCN, protocerebral connective; SEC, supraesophageal commissure; TC, tritocerebral commissure; VC, ventrocentral; VL, ventrolateral; VM, ventromedial.
(A–C) Starting from the ventral surface of the embryonic brain ventromedial (VM) and ventrolateral (VL) lineages in the protocerebrum express Hbn. In deeper sections, ventrocentral (VC) and dorsomedial (DM) expression is visible.
(D–F) Middle sections of the brain show the supraesophageal brain commissure with Hbn expressing cells in close association with the commissure (white arrowheads) and lineages dorsomedial (DM) and lateral (yellow arrowheads) in the protocerebrum as well as Hbn expression in the mushroom bodies (MB) and around the protocerebral connective (PCN).
(G–I) Hbn expression in the dorsal part of the brain is visible in a dorsocentral (DC) region, close to the optic lobes (OL) and near the tritocerebral commissure (TC) (white arrowheads).
(Scale bar: 20 μm.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

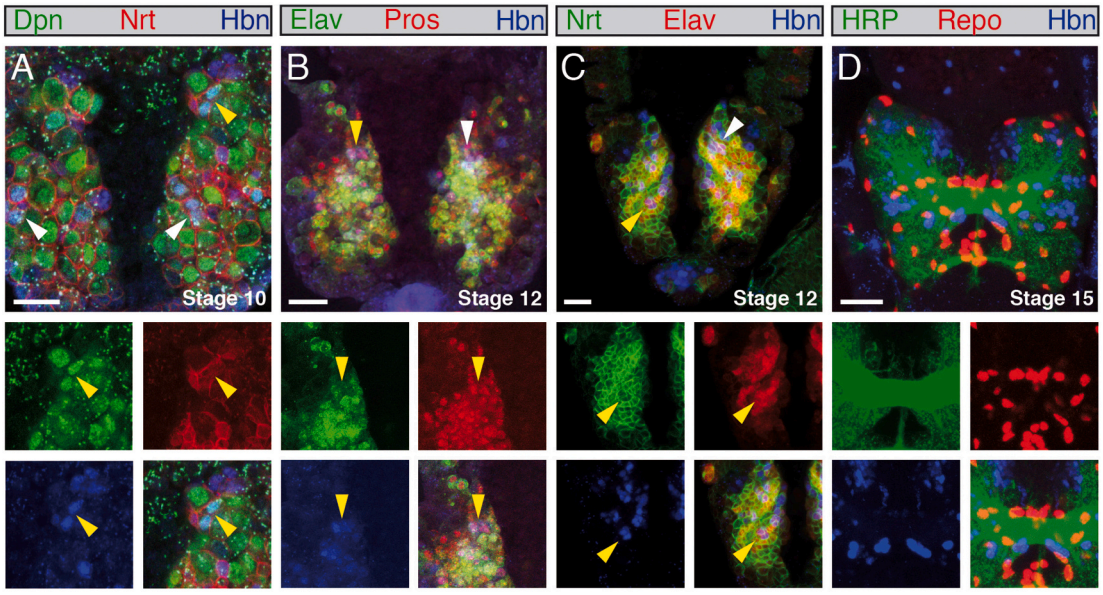

Fig. 3. Cell type identification of Hbn expressing cells in the developing embryonic brain.
Dorsal views of embryonic brains with the anterior ends of the embryos pointing towards the bottom. Anti-Nrt and anti-HRP antibodies were used to highlight primary neurons (Nrt) and sensory neurons, peripheral nerves and all fibre tract cells (HRP). Some regions with coexpression of the markers used are indicated by arrowheads. For better visualizations, the regions around the yellow arrowheads are shown in single channels.
(A) In a stage 10 embryo coexpression of the neuroblast marker Dpn (green) and Hbn (blue) was observed in several neuroblasts close to the midline below the procephalic neuroectoderm near the head-furrow and in the lateral Hbn-expressing zones (arrowheads).
(B) The GMCs show a Pros (red)-positive and a nuclear Elav (green)-negative staining. In a stage 12 embryonic brain, Hbn expression (blue) was detectable in some GMCs (arrowheads). These GMCs are spread over the dorsomedial and anterior part of the brain.
(C) Coexpression of Hbn (blue) and Elav (red)-positive cells indicates a rapid appearance of differentiated Hbn-expressing neurons in clusters widely spread over the entire brain (arrowheads) in a stage 12 embryo.
(D) The glial cell marker Repo (red) and Hbn (blue) show no coexpression indicating that Hbn is not expressed in glial cells in stage 15 embryonic brain.
(Scale bars: 20 μm.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development (Fig. 3D). The results demonstrate that Hbn expression in the protocerebrum occurs in neuroblasts, GMCs and neurons, but not in glial cells.

2.3. Generation of hbn mutants

To investigate the gene function of *hbn* we performed an EMS mutagenesis screen (Grigliati, 1986) to isolate point mutants from the 57B region of the second chromosome. By complementation analysis using a lethal P-element strain (KG02514) with an insertion 130 bp upstream of the *hbn* transcription start site, we identified two mutant strains (4028 and 15227) within our collection of lethal mutants, which might represent new *hbn* alleles. Sequencing of the whole *hbn* coding region of both strains revealed single C to T transitions, which induced a change from a glutamine codon to a stop codon in both cases verifying that the mutant strains 4028 and 15227 represent new *hbn* alleles, hereafter called hbn^{4028} and hbn^{15227} (Fig. 4A). Compared with the 409 aa long wild-type Hbn protein, the mutant versions were shorter, consisting of 201 aa including a part of the homeodomain for hbn^{4028} and only 87 aa in the case of hbn^{15227} (Fig. 4B). Based on antibody staining against Hbn, we were able to detect the longer Hbn^{4028} protein but not the short Hbn^{15227} protein (data not shown). For both new alleles hbn^{4028} and hbn^{15227} lethality was tested using balanced strains with GFP- marked CyO chromosomes. This procedure enabled the selection of homozygous mutant embryos. After 24 h of development, none of the hbn^{15227} homozygous embryos hatched, and after 48 h only a few escapers were visible (0.67%) which then died in the first larval stage (Fig. 4C). In the case of hbn^{4028}, more larvae hatched after 24 h (7.29%) and even more after 48 h (62.91%), but also in this case, no further development was observed and all the larvae died soon after hatching. These results indicate that the hbn^{15227} allele is slightly stronger than the hbn^{4028} allele, potentially due to the presence of a partial homeodomain in the hbn^{4028} allele. The two alleles were also analysed in a trans-heterozygous background with themselves and with the deficiency *Df (2R)Exel7166*, uncovering part of the 57B region of the second chromosome including *hbn*. In all combinations they showed embryonic lethality, thus confirming their identities as new *hbn* alleles.

2.4. Analysis of hbn^{15227} mutant embryos

To analyse the effect of a mutation in *hbn* we first used *in situ* hybridization to compare the distribution of the *hbn* mRNA from wild-type embryos with hbn^{15227} mutant embryos. Assuming that the point mutation in hbn^{15227} embryos had no effect on mRNA expression, hence a loss of expression in certain regions might provide a first hint about affected areas in *hbn* mutants. To identify mutant embryos unambiguously, they were preselected using a GFP-marked CyO balancer chromosome here and in all following experiments. In a lateral view of stage 9 wild-type embryos, *hbn* expression was visible in four discrete areas (Fig. 5A), whereas mutant hbn^{15227} embryos of the same stage showed expression in only one area, and the lateral and clypeolabral domains were missing (Fig. 5B). In stage 16, the *hbn* mRNA was primarily localized in the brain region in several strong spots in both hemispheres and their connecting bridge (Fig. 5C). In contrast, the *hbn* expression pattern in a mutant stage 16 embryo was clearly diminished, and only a few weak spots could be identified in the area of the hemispheres (Fig. 5D). This loss of *hbn* expression in defined regions in mutant hbn^{15227} embryos provides a first indication that cells in these regions may have been lost in these embryos. As *hbn* is expressed during early development in an anterior stripe in the head region similar to the gap genes (Cohen and Jürgens, 1990) it might also have an effect on head development. To examine this possibility we made cuticle preparations.

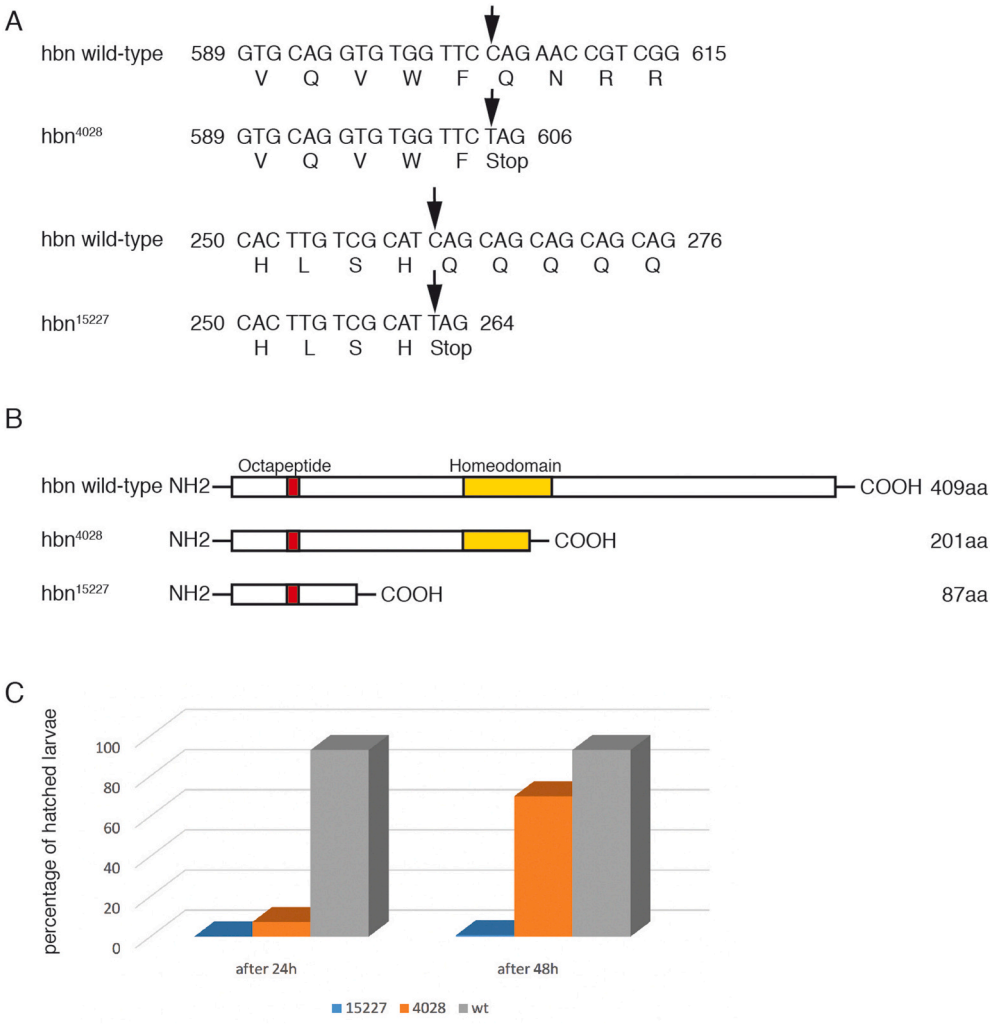

Fig. 4. Characterization and analysis of the mutant alleles *hbn*^{15227} and *hbn*^{4028}.
(A) Nucleotide and amino acid sequences in wild-type and mutant DNA. EMS induces C to T transitions in the coding region of *hbn*^{15227} and *hbn*^{4028} leading to the formation of stop codons.
(B) Schematic overview of the wild-type Hbn protein with the localization of octapeptide and homeodomain in comparison to the truncated mutant proteins of the *hbn*^{4028} and *hbn*^{15227} alleles.
(C) Lethality test of homozygous mutant animals of *hbn*^{15227} and *hbn*^{4028} compared with wild- type animals. Hatched larvae were counted 24 h and 48 h after egg laying.

A cuticle preparation of the head of a wild-type first instar larva is shown in Fig. 5E. Clearly visible structures from anterior to posterior are the mouth hooks, labrum, ekto- and epistomal sclerite, H-piece and the cephalopharyngeal skeleton consisting of the lateralgräte, dorsal bridge, dorsal arms, vertical arms and vertical plate. Most of these structures can also be identified in *hbn* mutants except for the labrum and the epistomal sclerite, the dorsal bridge has a different shape (Fig. 5F, arrowheads). Fate map studies in blastoderm embryos (Jürgens et al., 1986) have shown that all the structures that are missing or altered in *hbn* embryos derive from anteriorly located regions where *hbn* is expressed in early embryos.

2.5. Characterization of the neural phenotypes of the mutant hbn alleles

For a more detailed analysis of potential neural phenotypes of *hbn* mutants we performed antibody staining with wild-type embryos as well as *hbn*^{15227} and *hbn*^{4028} mutant embryos using the common neuronal markers HRP and Nrt as well as FasII (Bastiani et al., 1987), which labels only subsets of the axon fascicles of all commissures. In wild-type stage 16 embryos, the complete brain was stained with Nrt (Fig. 6A), whereas FasII staining highlighted the commissures including the three tracts of the supraesophageal commissure (Fig. 6B, white arrowheads). In the *hbn*^{15227} mutants, the protocerebral part of the brain appeared reduced in size, and the commissure between both hemispheres was missing (Fig. 6A′, white arrowhead). The FasII staining revealed a loss of the three FasII-positive fibre tracts interconnecting the brain hemispheres in *hbn*^{15227} mutants (Fig. 6B′, white arrowhead) compared with the wild-type (Fig. 6B). In addition P4 founders were altered in size and shape (Fig. 6B & B′, yellow arrowheads), and the optic lobes were dislocated anteriorly to the protocerebrum (Fig. 6B & B′, red arrowhead) similar to *otd* mutant brains (Younossi-Hartenstein et al., 1997). Comparable experiments with the second *hbn* mutant *hbn*^{4028} showed similar results. HRP-stained embryonic brains showed a tremendous reduction of the anterior part of the protocerebrum (Fig. 6C & C′, yellow arrowheads) and loss of the supraesophageal brain commissure (Fig. 6C & C′, white arrowheads). The protocerebral connectives (PCN) seemed to be ablated or reduced (Fig. 6C′ & D′, yellow arrowheads) and the optic lobe dislocation was again clearly visible (Fig. 6C′ & D′, red arrowheads). In about 20% of the cases a very thin commissure was still visible in *hbn*^{4028} mutant embryos; this allele was thus a little bit weaker in comparison to *hbn*^{15227} which is also in agreement with the larger amount of hatching larva in the case of *hbn*^{4028}. The described phenotypic results were obtained for all possible transheterozygotes (data not shown).

During our analysis of DRx function in mushroom bodies (Kraft et al., 2016), we found that Hbn was coexpressed with DRx in mushroom body neuroblasts in the embryonic brain. This observation was recently confirmed by Curt et al., 2019. The reduction of this part of the protocerebrum in mutant embryos provided the first hints that the embryonic mushroom body neuroblasts and their embryonic lineages might be affected in *hbn* mutants. To examine this possibility, we used the mushroom body marker 238Y-GAL4, which identifies two mushroom body neuroblasts and their progenies in the embryonic brain

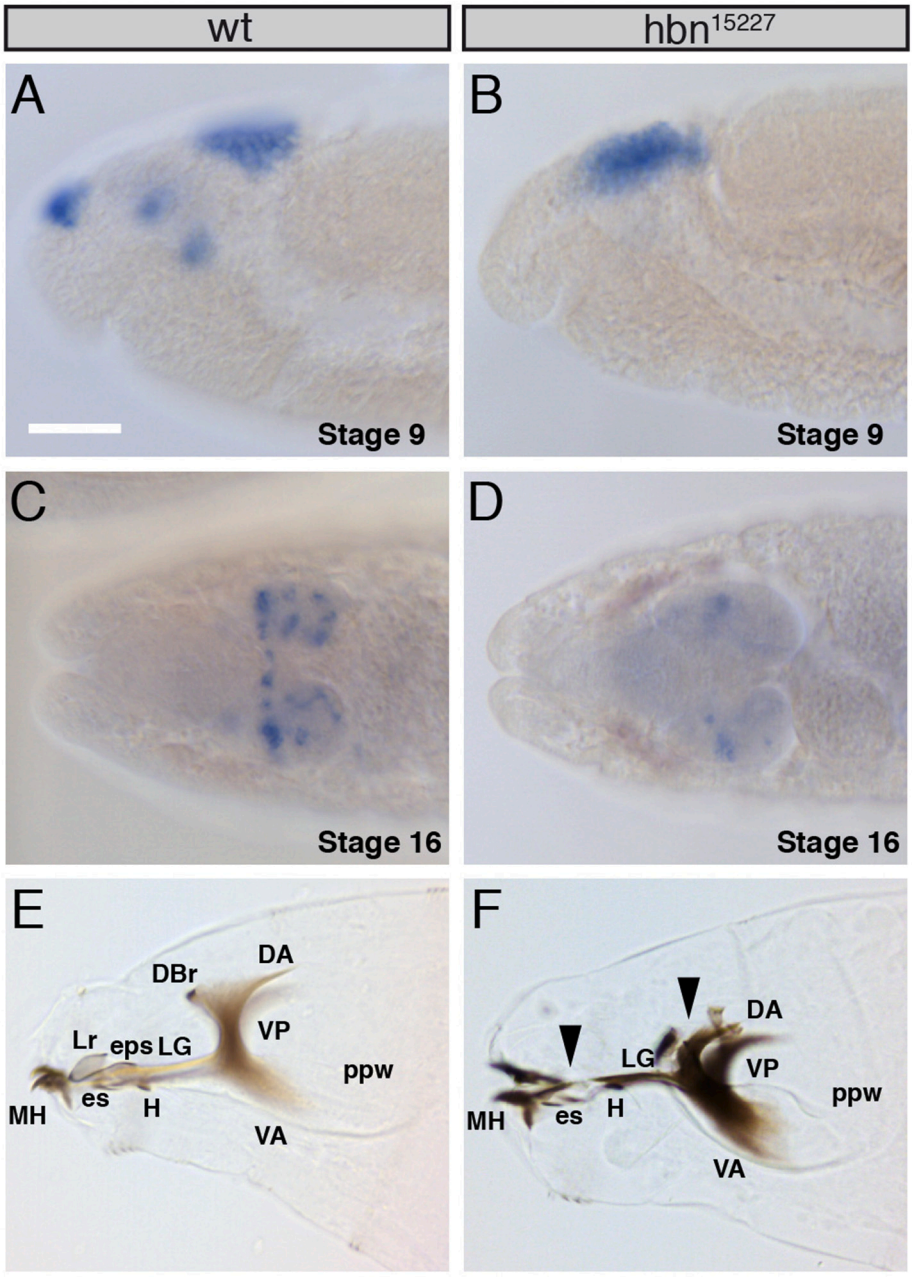

Fig. 5. Differences between wild-type and *hbn*^{15227} mutant embryos and larvae.
Hbn in situ hybridization in wild-type (A, C) and *hbn*^{15227} mutant embryos (B, D). Only the anterior parts of the embryos are shown. In all views the anterior end is to the left. In stage 9 embryos (lateral views) four *hbn* expression domains are visible in a wild-type embryo (A) compared with only one central expression domain in a mutant embryo (B). In stage 16 embryos (dorsal views), *hbn* expression is reduced in the brain hemispheres of a mutant embryo (D) compared with a wild-type embryo (C). Cuticle preparations of wild-type (E) and *hbn*^{15227} mutant larva (F) in lateral views, anterior is to the left. Abbreviations are according to Jürgens et al. (1986). DA, dorsal arm; DBr, dorsal bridge; eps, epistomal sclerite; es, ectostomal sclerite; H, H-piece; LG, lateralgräte; Lr, labrum; MH, mouth hooks; ppw, posterior wall of pharynx; VA, ventral arm; VP, vertical plate. Regions of missing structures in the mutant larva compared with the wild-type larva are indicated by arrowheads.
(Scale bar: 20 μm.)

(Tettamanti et al., 1997; Kurusu et al., 2000). In wild-type embryos, the 238Y-GAL4 marker clearly indicated these mushroom body lineages in the embryonic brain (Fig. 7A, arrowheads). In *hbn*^{15227} mutant embryos, the strongly reduced protocerebrum was visible and the 238Y-GAL4 marker was not expressed in the region where the mushroom body lineages were supposed to be (Fig. 7B, arrowheads), clearly showing a loss of corresponding cells in *hbn* mutant embryos.

2.6. Formation of the supraesophageal brain commissure with participation of an Hbn-positive cellular bridge

The very strong *hbn* phenotype concerning the supraesophageal commissure prompted us to analyse the formation of the supraesophageal commissure in more detail focusing on the role of Hbn in this process. Earlier studies have shown that protrusions of both brain hemispheres extend towards the midline and form an interhemispheric cell bridge (Therianos et al., 1995). Fibre tract founder clusters and their axons forming a system of pioneer tracts (DT, P1, P2l, P2m, P3l, P3m, P4l, P4m, P5l and P5m) in a defined temporal order (Nassif et al., 1998) were identified using FasII as a marker. Interestingly, Hbn was expressed in most of these pioneer cells (Fig. S2). Among them, P2l and P2m particularly serve as pioneer cells of the supraesophageal brain commissure, with axons growing to the dorsal midline and fasciculating with their contralateral counterparts (Nassif et al., 1998). We analysed commissure formation in association with the observed Hbn positive P2m founder cells by tracking the Hbn/FasII-positive pioneer cells in an HRP-marked background at different time-points between stage 12 and stage 14 when the commissure formation occurs. At early stage 12, an accumulation of 4 Hbn/FasII-positive cells, including the P2m cell, protruded from both sides towards the midline (Fig. 8A, white arrowheads), and several HRP-positive growth cones (Fig. 8A, yellow arrowhead) preceded the innermost Hbn/FasII-positive P2m cell; in contrast, the other three cells were not FasII-positive. The protrusions extending towards the midline with the Hbn/FasII-positive cell in front (Fig. 8B,

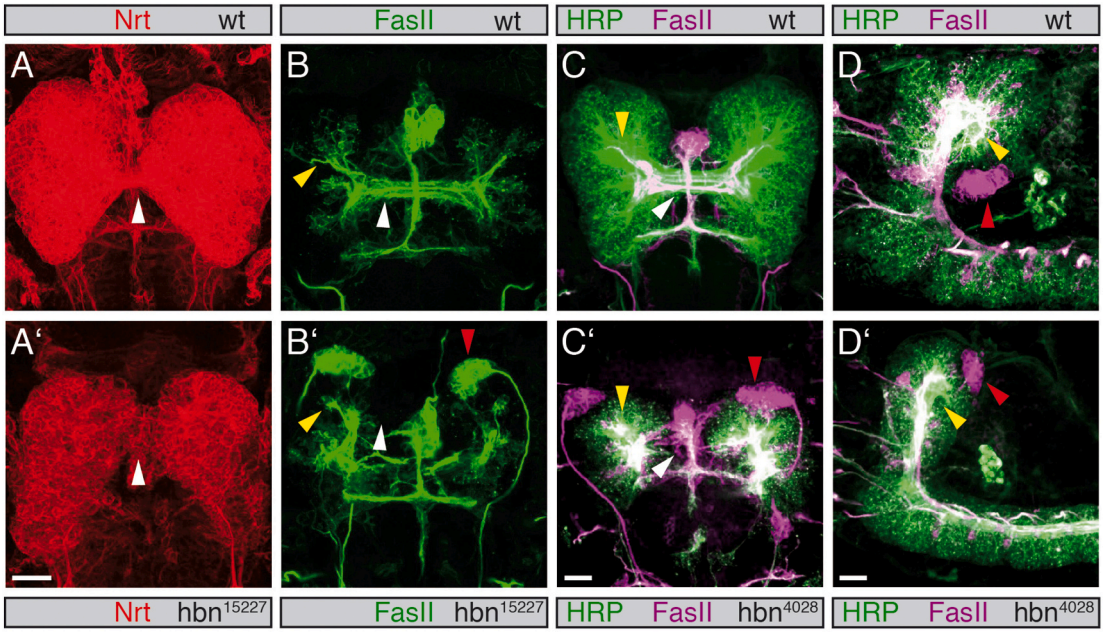

Fig. 6. Comparison of wild-type and mutant *hbn*^{15227} and *hbn*^{4028} brains with Nrt and FasII as neuronal markers.
Staining of stage 16 wild-type (A) and *hbn*^{15227} mutant (A′) embryonic brains with Nrt (red) to illustrate the surface of the embryonic primary neuronal lineages. In the mutant, the brain size is reduced and the brain commissure deleted (arrowheads). The brain-associated pattern of FasII (green) in a wild-type (B) and a *hbn*^{15227} mutant brain (B′) in stage 16 embryos. In the *hbn*^{15227} mutant brain, the commissure is missing (white arrowheads), P4 founders are altered in size and shape (yellow arrowheads) and the optic lobe is dislocated to the anterior tip of the protocerebrum (red arrowhead). A mutant *hbn*^{4028} brain (C′) displays a reduction in size and shape (yellow arrowhead), as well as the loss of commissural tracts (white arrowheads) in comparison to a wild-type (C) stage 16 embryonic brain; dislocation of the optic lobe is also visible (red arrowhead). In contrast to the wild-type brain (D), both the P4 FasII tract in the protocerebral connective (yellow arrowheads) and optic lobe (red arrowheads) are not positioned correctly in the mutant (D′). (A–C′) Dorsal view, anterior is down. (D and D′) Lateral view, anterior is to the left.
(Scale bars: 20 μm; A–D and B′ like A′.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

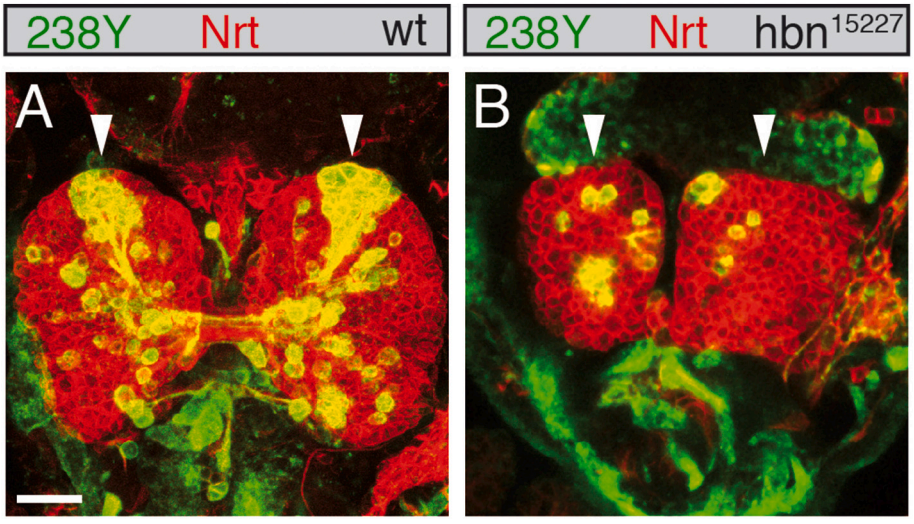

Fig. 7. Embryonic mushroom body lineages are lost in *hbn*^{15227} mutant brains.
Staining of stage 16 wild-type (A) and *hbn*^{15227} mutant (B) embryonic brains with Nrt (red) and the mushroom body specific marker 238Y-GAL4 (green) (dorsal views). In a wild-type brain (A), the location of the embryonic mushroom body lineages is indicated by the yellow coexpression of Nrt and 238Y-GAL4 (arrowheads). The *hbn*^{15227} mutant brain (B) shows a strong reduction in size and no visible marker expression in the embryonic mushroom body lineages (arrowheads).
(Scale bar: 20 μm.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

white arrowheads) were associated with HRP-positive growth cones (Fig. 8B, yellow arrowhead). In the next step, these HRP-positive growth cones met at the midline (Fig. 8C, yellow arrowhead) and fused (Fig. 8D, green arrowhead). The P2m founder cells from both sides joined and the HRP-positive commissural part began to grow (Fig. 8E, green arrowhead) until a complete cellular bridge composed of HRP and Hbn/FasII-positive cells was build (Fig. 8F). In stage 14 the Hbn/FasII-positive cells remained in close association at the midline during the thickening of the HRP commissural section. The formation of FasII-positive ventral and dorsal commissural tracts as well as lateral protocerebral tracts was then clearly visible (Fig. 8G, H, red arrowheads). During the late stage 14, Hbn/FasII-positive pioneer cells and associated Hbn positive neurons were relocated away from the midline towards both brain hemispheres to their original positions (Fig. 8I, red arrowheads).

2.7. *Apoptosis causes the smaller brain phenotype in hbn mutants*

Finally, we wanted to determine how the *hbn* phenotype was established. To explain the loss of the protocerebral structures in the mutant brains, two different possibilities were considered. The first was a reduced rate of cell proliferation, and the second was an increased amount of apoptosis or both of these events together. Since apoptosis is

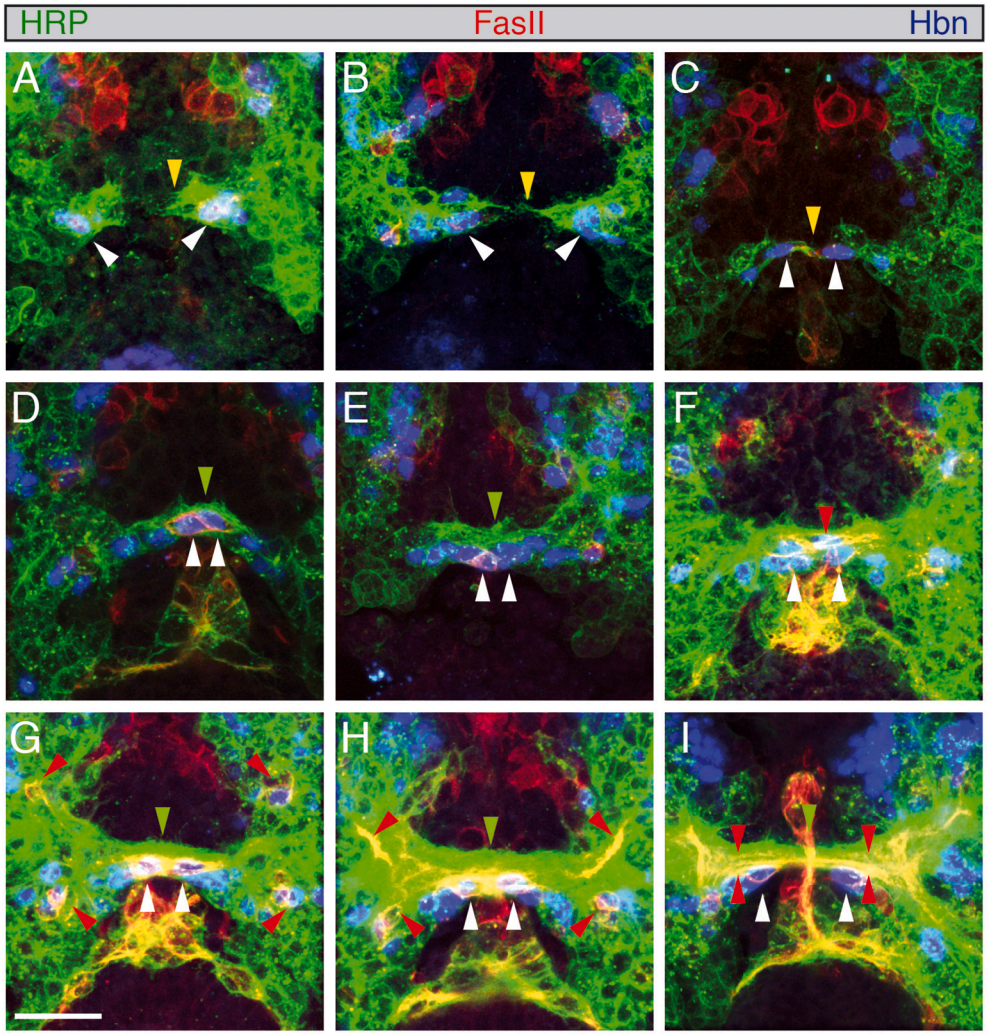

Fig. 8. Formation of the supraesophageal brain commissure with the participation of an Hbn-positive cellular bridge.
Staining of embryonic brains from stage 12 (A–C), stage 13 (D–F) and stage 14 (G–I) using the antibodies anti-HRP (green), anti-FasII (red) and anti-Hbn (blue). All views are from the dorsal aspect, with the anterior end of the embryo pointing down.
(A) In a stage 12 embryo, the pre-established protrusions of both hemispheres are enlarged, and several HRP-positive growth cones (yellow arrowhead) precede the Hbn/FasII-positive P2m cell (white arrowheads).
(B) HRP-positive growth cones touch each other at the midline (yellow arrowhead). Hbn-positive cells are lined up along the protrusions of the hemispheres (white arrowheads).
(C) Hbn/FasII-positive cells attach at the centre of the midline and remain close together during establishment of the complete Hbn-positive cell bridge.
(D) At stage 13, the Hbn/FasII founder cells (white arrowheads) are in close association with the midline centre, and the commissural tracts start to cross the midline, as indicated by HRP staining (green arrowhead).
(E) The HRP connections of the protocerebral brain commissure start thickening (green arrowhead) and the Hbn-positive cellular bridge is fully established with two central Hbn/FasII-positive cells (white arrowheads).
(F) FasII-positive commissural tracts appear, and the Hbn/FasII-positive midline cells are clearly visible flanking the centre of the midline (white arrowheads) holding strong FasII fascicles (red arrowhead).
(G) The Hbn/FasII-positive founder cells remain at the midline (white arrowheads). The HRP portion of the commissure is enlarged (green arrowhead), and the ventral and dorsal commissural FasII-positive tracts (red arrowheads) emerge in a stage 14 embryo.
(H) Hbn/FasII-positive cells relocate from the midline to the hemispheres (white arrowheads) and different FasII-positive commissural tracts can be distinguished (red arrowheads).
(I) Hbn/FasII-positive cells almost reach their final position at the beginning of the commissure, while the supraesophageal commissure is still growing in size.
(Scale bar: 20 μm.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the more likely reason for the brain phenotype and together with our recent finding that apoptosis is responsible for the missing hindgut structures in *orthopedia* mutants (Hildebrandt et al., 2020) we first analysed apoptosis in wild-type and mutant embryonic brains. We used the apoptosis marker Dcp-1 (Florentin and Arama, 2012) together with Nrt to visualize the surface of the developing brains. In a dorsal view of a wild-type brain of embryonic stage 11, several apoptotic cells were visible scattered over the brain (Fig. 9A). In contrast, in a mutant brain of the same stage, an increase in apoptosis was already visible in several regions (Fig. 9A′, arrowheads). At stage 13 massive apoptosis was visible predominantly in the anterior part of the mutant brain (Fig. 9B′, arrowheads) compared to the wild-type brain (Fig. 9B). These results could also be confirmed in lateral views in which we detected a very large number of apoptotic cells at the anterior tip of the mutant brain (Fig. 9C′, arrowhead) compared with the wild-type brain (Fig. 9C).

To investigate proliferation in wild-type brains we performed a co-staining of Hbn with the proliferation marker phospho-Histone H3 (Bello et al., 2006) in a HRP background to highlight the brain (Fig. S3).

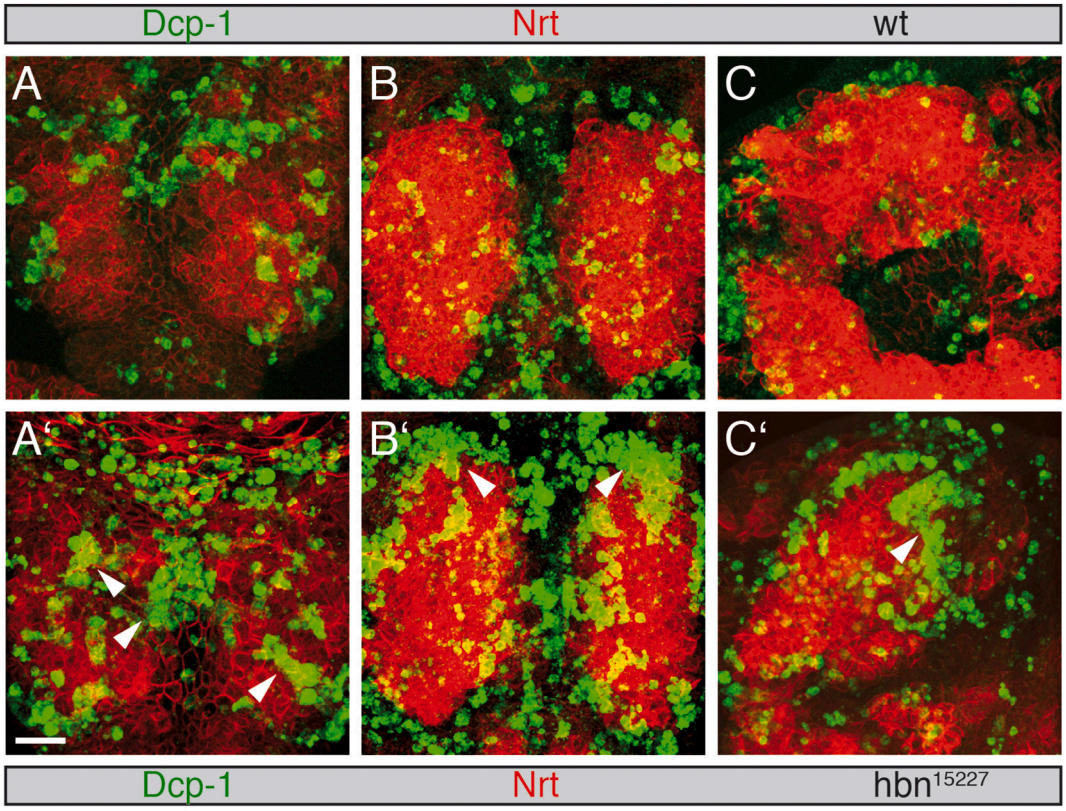

Fig. 9. Apoptosis in wild-type and *hbn*^{15227} mutant embryonic brains.
Comparison of apoptosis in wild-type (A–C) and *hbn*^{15227} (A′–C′) mutant brains of embryonic stage 11 (A, A′) and stage 13 (B, B′, C, C′). Nrt (red) marks neuronal cells, and Dcp-1 (green) highlights apoptotic cells.
Dorsal views show a strong increase in apoptosis in the mutant brain (A′) already at stage 11 (arrowheads) compared with the wild-type brain (A). At stage 13 apoptosis is increasing in the mutant brain (B′) especially at the anterior tip of the protocerebrum (arrowheads) compared to the wild-type brain (B). This effect is also visible in lateral views of a wild-type brain (C), compared with a mutant brain (C′), again at the anterior tip of the protocerebrum (arrowhead).
(Scale bar: 20 μm.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Here we show Hbn proliferating cells in the brain between stage 11–16 (Fig. S3A–F), the time window when the brain is formed. These cells are located at the anterior tip of the protocerebrum as well as some dorsal and ventral surface regions. Since from stage 11 on the first areas of the brain are affected in *hbn* mutants by emerging notches, a corresponding analysis is not possible in the mutant and we do not see many colocalization of Hbn and pH3 because Hbn expressing cells get lost and the brain phenotype will be established. Therefore we cannot rule out, that reduced proliferation is also contributing to the phenotype, but we favour that apoptosis rather than missing cell proliferation is responsible for the *hbn* brain phenotype.

3. Discussion

In this paper, we analysed the function of the transcription factor Homeobrain during embryonic brain development. Hbn shows a very dynamic expression pattern during development, mainly in the protocerebrum but also in the deutocerebrum and the tritocerebrum. In the protocerebrum the expression can be detected in the founder cell cluster for the supraesophageal commissure, the mushroom bodies and several other regions. The expression is visible in approximately 13 cells, which are most likely neuroblasts, followed by GMCs and neurons. Starting from a population of approximately 108 neuroblasts per hemisphere, the complete brain is constructed later (Urbach and Technau, 2003). Most of them show the type I division mode except for 8 type II neuroblasts and 4 MBNBs (Curt et al., 2019). The 8 type II neuroblast are of great interest since they produce large lineages of up to 400 neurons each during the larval stage (Knoblich, 2010) and generate a major portion of the adult central complex neurons (Bayraktar et al., 2010). Hbn is expressed in all type II lineages in the larval brain (data not shown), but for a long time it was not clear whether type II neuroblasts already existed in the embryo. It was recently shown that they are generated in the embryo between stage 11 and 14 and are present in an anterior dorsomedial (ADM) cluster and a posterior dorsomedial (PDM) cluster of three neuroblasts each and a dorsolateral (DL) cluster of two neuroblasts (Walsh and Doe, 2017; Álvarez and Díaz-Benjumea, 2018). In a search for transcription factors driving the anterior expansion of the embryonic brain during CNS development *hbn* and its neighbouring genes *DRx* and *otp* were analysed. It was shown that Hbn is together with DRx expressed in PDM and DL-type II neuroblasts, five of the eight type II neuroblasts (Curt et al., 2019). A mutant analysis of *DRx* and *otp* alone did not result in a proliferation effect, potentially due to genetic redundancy, but removal of all three genes using a deletion resulted in a reduced number of type II neuroblasts and daughter cells (Curt et al., 2019). This finding clearly argues for a major function of Hbn in PDM and DL neuroblasts and it will be of interest to analyse this in detail in the future using our *hbn* mutants. The expression of Hbn in mushroom bodies described herein was also observed by Curt et al., 2019 using OK107-Gal 4 as a mushroom body marker. There are several key regulators of mushroom body development including the transcription factors Dachshund (Dac), Eyeless (Ey) and Tailless (Tll) (Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000; Kurusu et al., 2009). Recently, we showed that DRx also controls mushroom body neuroblast growth and proliferation (Kraft et al., 2016). The present analysis showed that mushroom body progenitor cells are also lost in *hbn* mutants; therefore, Hbn is another important factor in early events of mushroom body development in *Drosophila*. Mushroom

body progenitor development is a good example for a combinatorial expression of transcription factors driving the establishment of different lineages (Kunz et al., 2012). A combination of homeobox genes is also controlling patterning processes of the *Drosophila* ventral nervous system (see Estacio-Gómez and Díaz-Benjumea, 2014 for review) and primary neuronal cluster expression in the brain (Sprecher et al., 2007). Even neuron classes or individual neurons can be described individually by a specific combination of expressed homeodomain proteins as shown recently in *Caenorhabditis elegans* (Reilly et al., 2020). This is most likely also the case in *Drosophila* and Hbn might be one descriptor of specific neurons in the brain.

In addition to its function in various lineages in the embryonic brain, one major function of Hbn is to establish the supraesophageal commissure. In *hbn* mutants the commissure is completely absent. We analysed the formation of the commissure in detail and found that Hbn was expressed in the fibre tract founder cells P2l and P2m. When protrusions of the brain were visible, Hbn was expressed in four cells of the protrusion, among them the P2m founder cells. The innermost of these cells in the protrusion was Hbn/FasII-positive, and its growth cone together with neighbouring growth cones extended to the midline. The other three Hbn-positive cells were lined up in a row. When the growth cones met at the midline, the interhemispheric bridge was built and the Hbn/FasII-positive cells were visible next to each other at the midline. Following these early events constructing the ventral commissural tract, the commissure is enlarged through additional interhemispheric dorsal tracts emerging from P3l and P4l founders (Nassif et al., 1998) where Hbn is most likely also expressed. Formation of the interhemispheric bridge results from the HRP-positive growth cones from both sides and not by pioneer cells or by an active migration process. Building of the initial protrusions is not *hbn*-dependent since the protrusions were also present in *hbn* mutants. In embryos of the weaker hbn^{4028} allele, a very thin commissure was sometimes visible, which might be built *hbn*-independent, but the expression of Hbn in the pioneer cells seemed to be necessary to attract additional interhemispheric fibre tracts. We have experimental evidence for this assumption since the addition of an extra copy of a 20 kb fragment covering the genomic region of *hbn* in the genome *via* Pacman transgenesis rescued the *hbn* phenotype but also leads to a thickening of the commissure at early stages in the wild-type background. The initial events in building the commissure with the help of the pioneer cells and the later events of thickening of the commissure with the participation of additional tracts are regulated independently since we identified two different regulatory elements of *hbn* that label the different tracts of the commissure individually using reporter assays (Hildebrandt et al., in preparation). These regulatory elements might help to analyse both processes in more detail and to ascertain whether the later events of thickening of the commissure truly depend on the earlier events of commissure formation.

A phenotype similar to the weak *hbn* phenotype has been found in *commissureless* (*comm*) mutants. Comm is an intracellular sorting receptor that prevents the Robo receptor from being delivered to the growth cone during axon guidance events (Keleman et al., 2002; Myat et al., 2002). In *comm* mutants, the Hbn-positive founder cells are not mobilized, and only a thin commissure is built, comparable to the weaker *hbn* phenotype. A complete absence of the supraesophageal commissure and a missing interhemispheric bridge can also be observed in *jing* mutants. Jing is a zinc finger transcription factor that is expressed in the embryonic CNS midline glia, in tracheal cells, in the embryonic brain during early and late stages of differentiation as well as in oocytes, where it fulfils a function during the initiation of border cell migration (Liu and Montell, 2001; Sedaghat and Sonnenfeld, 2002; Sedaghat et al., 2002). *Jing* function in the embryonic brain is required for the differentiation of Repo, Castor and Sim-positive cells (Sedaghat and Sonnenfeld, 2002).

One emerging question during our analyses concerned how the *hbn* phenotype could be explained. One possibility is a loss of proliferation, and the other possibility is cell death or both. Our results clearly indicated that massive cell death occurred in the protocerebrum starting from stage 11. This cell death was visible before formation of the commissure and affected mainly the anterior and medial regions of the protocerebrum where Hbn is active, but we cannot rule out that a loss of proliferation also contributes to the *hbn* phenotype. This phenotype with substantial loss of parts of the protocerebrum is similar to *otd* and *wingless* (*wg*) phenotypes in the brain. In *tll* mutants the complete protocerebrum including the supraesophageal commissure is absent (Strecker et al., 1986), in *otd* mutants, the protocerebral brain neuromere is eliminated (Hirth et al., 1995), and in *wg* mutants, one half of the protocerebrum is deleted by apoptosis in later embryonic stages (Richter et al., 1998). These phenotypes are similar to those of the *hbn* mutants.

In summary, the results presented in this study demonstrate, that Hbn is an important factor in *Drosophila* embryonic brain development that plays a role in the generation of the supraesophageal commissure, mushroom body progenitor cells and various brain structures. It will be interesting to analyse the function of Hbn in mushroom body development and type II cell lineages, as well as later functions of Hbn during larval development, using our newly generated *hbn* alleles in the future.

4. Material and methods

4.1. Fly strains

The following fly strains were used: yw^{67c3}; hbn^{4028}/CyO otp lacZ, hbn^{4028}/CyO GFP; hbn^{15227}/CyO otp lacZ, hbn^{15227}/CyO GFP; UAS-nlacZ (S. Hayashi); 238Y-GAL4 (J. Armstrong). The following stocks were obtained from the Bloomington *Drosophila* Stock Center: UAS-mCD8::GFP (Bl 5130), Df(2R) Exel7166 (Bl 7998), KG02514 (Bl 13750).

4.2. Amplification and cloning of the coding region of hbn alleles

Amplifications of the coding region were performed using genomic DNA from fly stocks hbn^{4028} and hbn^{15227}. For polymerase chain reactions Taq Polymerase from ThermoFisher Scientific (Waltham, Massachusetts, USA) was used according to supplier's instructions. PCR products were sequenced by Starseq (Mainz, Germany). Regions showing sequence alterations compared with the wild-type sequence were again PCR-amplified using more closely located primers. The primers 4028A (5′-CAGATTGATAGATTGGGAAATTGTTCG-3′) and 4028B (5′-ATTTGATGGGGTGGATTCGTGAGATGGG-3′) were used for hbn^{4028} DNA and the primers 15227A (5′-TGAGGATA-TAACTACTTCAGCCACAATTG-3′) and 15227B (5′-GAGATCATGGC-CATTGTTCAGAC-3′) for hbn^{15227} DNA. The PCR products were subcloned into the TOPO vector pCR2.1 (ThermoFisher Scientific, Waltham, Massachusetts, USA), and at least 10 individual clones from each PCR product cloning were checked by sequencing. Since the hbn^{4028} and hbn^{15227} DNA was generated from heterozygous flies approximately 50% of the clones showed the wild-type sequence and 50% the altered sequence due to the point mutations.

4.3. Hbn antibody preparation

To generate the guinea-pig anti-Hbn antibody, a 455 bp fragment from the *hbn* cDNA (amino acids 1–151) was PCR-amplified with primers hbn3 (5′-ATGATGACCACGACGACC-3′) and hbn rev (5′-TTACGCGGCCTCTCCATA-3′) and subcloned into the TOPO vector pCR2.1 (ThermoFisher Scientific, Waltham, Massachusetts, USA). The *hbn* fragment was excised with *Eco*RI and cloned in frame into the pGEX-4T1 expression vector (GE Healthcare). The fusion protein of glutathione-S-transferase and Hbn was purified as previously described (Smith and Johnson, 1988). Immunization of guinea-pigs was performed by Pineda Antibody Service (Berlin).

4.4. Immunocytochemistry

Embryos were dechorionated, fixed and labeled according to Therianos et al., 1995 and staged according to Campos-Ortega and Hartenstein (1997). Primary antibodies were guinea-pig anti-Hbn antibody (1:1000), goat FITC-conjugated anti-HRP antibody (1:100) (ICN), rabbit anti-β-galactosidase antibody (1:1000) (Promega), mouse anti-β-galactosidase antibody (1:1000) (Upstate), rabbit anti-phospho-Histone H3 (1:200) (Santa Cruz Biotechnology), guinea pig anti-Dpn antibody (1:500) (a gift from Jürgen Knoblich), rabbit anti-Dcp-1 antibody (1:100) (Cell Signaling Technology), and rabbit anti-pH3 antibody (1:200) (Upstate). Mouse anti-En antibody (1:10), rabbit anti-Elav antibody (1:30), mouse anti-Repo antibody (1:10), mouse anti-Pros antibody (1:10) and mouse anti-FasII antibody (1:4) were obtained from the Developmental Studies Hybridoma Bank, Iowa. Secondary antibodies were Alexa Fluor 488, 568 and 647 goat-anti-mouse IgG (H + L) antibodies, and 568 goat anti-rabbit, biotinylated goat anti-guinea-pig IgG (H + L) antibody (ThermoFisher Scientific, Waltham, Massachusetts, USA), all used at a 1:1000 dilution. For the detection of the guinea pig anti-Hbn, mouse anti-En and mouse anti-Pros antibodies served the TSA™ FITC, Cyanin3 and Cyanin5 System (Perkin Elmer, Waltham, Massachusetts, USA). Stained embryos were mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, Canada).

4.5. Microscopy

For fluorescence microscopy two different microscopes were used, a ZEISS LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) with a 40× objective and a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) with a HyD detector and a constant laser speed of 8000 Hz or a variable detector using 400 Hz with a 63× objective. Optical sections from 0.5 µm up to 1 µm intervals were acquired. Captured images from optical sections were arranged and processed using FIJI and ImageJ (NIH. Md., USA) and Adobe Illustrator CS6 (Adobe Systems, San Jose, CA, USA). For bright field and DIC microscopy we used an Olympus BX 61 microscope (Olympus, Hamburg, Germany).

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CRediT authorship contribution statement

Dieter Kolb: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing, Visualization
Petra Kaspar: Conceptualization, Methodology, Validation, Investigation, Data curation
Christine Klöppel: Investigation, Data curation, Writing, Visualization
Uwe Walldorf: Conceptualization, Validation, Data curation, Writing - Review & editing, Visualization, Supervision.

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