TITLE

A targeted genetic screen identifies crucial players in the specification of the *Drosophila* abdominal Capaergic neurons

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Total character count: 49053

Figures: 6

Supplemental Figures: 3

RUNNING TITLE

Neuropeptidergic cell fate specification

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SUMMARY

The central nervous system contains a wide variety of neuronal subclasses generated by neural progenitors. The achievement of a unique neural fate is the consequence of a sequence of early and increasingly restricted regulatory events, which culminates in the expression of a specific genetic combinatorial code that confers individual characteristics to the differentiated cell. How the earlier regulatory events influence postmitotic cell fate decisions is beginning to be understood in the *Drosophila* NB 5-6 lineage. However it remains unknown to what extent these events operate in other lineages. To better understand this issue, we have used a very highly specific marker that identifies a small subset of abdominal cells expressing the *Drosophila* neuropeptide Capa: the ABCA neurons. Our data support the birth of the ABCA neurons from NB5-3 in a *cas* temporal window in the abdominal segments A2-A4. Moreover, we show that the ABCA neuron has an ABCA sibling cell which dies by apoptosis. Surprisingly, both cells are also generated in the abdominal segments A5-A7, although they undergo apoptosis before expressing Capa. In addition, we have performed a targeted genetic screen to identify players involved in ABCA specification. We have found that the ABCA fate requires *zfh2*, *grain*, *Grunge* and *hedgehog* genes. Finally we show that the NB 5-3 generates other subtype of Capa-expressing cells (SECAs) in the third suboesophageal segment, which are born during a *pdm*/*cas* temporal window, and have different genetic requirements for their specification.

KEY WORDS

Drosophila, progenitor neuroblasts, temporal genes, neuropeptidergic cell identity, CAPA

1. INTRODUCTION

The central nervous system (CNS) displays a daunting cellular diversity, as well as tremendous complexity in cellular connectivity. To generate this neural diversity at the precise time and place, while establishing the correct connections, developing animals need to orchestrate expression of large numbers of regulatory genes with great temporal and spatial precision. A large amount of work during the last two decades has been dedicated to understanding the developmental mechanisms controlling neuronal specification. However, many questions about these developmental mechanisms remain

poorly understood. Given the relative simplicity of its CNS, *Drosophila* provides a very attractive system to tackle these questions.

The *Drosophila* CNS is usually subdivided into the brain and ventral nerve cord (VNC). The VNC arises from the neuroectoderm, a sheet of cells located in the ventral–lateral region of the embryo (reviewed in (Skeath and Thor, 2003). The neuroectoderm undergoes the action of the patterning genes along the anterior-posterior and dorsal-ventral axes. As a result of these processes, a checkerboard pattern of neural equivalence groups is generated. From these cell groups, individual cells delaminate to become the progenitor cells of the CNS, the neuroblasts (NBs). The delamination process occurs in five sequential waves and results in the formation of an invariant pattern of 30 NBs per hemisegment (Campos-Ortega, 1985), with each NB acquiring a unique fate based on its position in the grid (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). After segregation, NBs undergo a series of asymmetric cell divisions, ‘‘budding’’ off secondary progenitor cells denoted ganglion mother cells (GMCs), that in turn typically divide one final time to generate neurons and/or glia (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Doe and Technau, 1993). Each NB has a unique and stereotypic identity, as revealed by the size of its lineage (from two to 40 cells) and by the types of neurons and glia generated (Bossing et al., 1996; Schmid et al., 1999; Schmidt et al., 1997). Each typical hemisegment contains 30 NBs that delaminate from the ectoderm in seven distinct rows (Broadus et al., 1995). NBs generate a large pool of GMCs, which in turn produce about 400 postmitotic neurons and glia.

A well-defined cascade of transcription factors, the temporal gene cascade of *hunchback-kruppel-pdm-castor-grainyhead*, is expressed in a sequential fashion by most, if not all, CNS progenitors, and controls distinct ‘‘competence windows’’ in NBs over time (Brody and Odenwald, 2000; Cleary and Doe, 2006; Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006; Isshiki et al., 2001; Kambadur et al., 1998; Novotny et al., 2002; Pearson and Doe, 2003; Tran and Doe, 2008; Tsuji et al., 2008). These transcription factors are sequentially expressed in the NB and act to specify temporal windows in which the different GMCs are produced. In addition, other genes must be specifically expressed in subsets of NBs, GMCs and neurons for controlling the individual characteristics of differentiated neurons. However, comprehensive understanding of how spatial and temporal cues are translated into combinatorial

regulatory codes of cell fate determinants is limited to just a few cases (Allan et al., 2005; Allan et al., 2003; Baumgardt et al., 2009; Baumgardt et al., 2007; Benito-Sipos J., 2010; Karlsson et al.; Miguel-Aliaga et al., 2004; Miguel-Aliaga and Thor, 2004; Miguel-Aliaga et al., 2008). Apart from these exceptions, the genes involved in specification of most neurons are largely unknown, and there are too few examples in which the progenitor NB of a neuron, the temporal gene window, and the genetic mechanisms underlying its specification, are known. Addressing these fundamental issues requires a highly selective cell type–specific marker for unequivocally identifying specific sub-sets of neurons.

We have taken advantage of a highly restricted marker in the *Drosophila* VNC, an antibody against the Capability (Capa) pre-propeptide (Kean et al., 2002), to gain further knowledge about how early regulatory events culminate in the expression of a specific genetic combinatorial code in different lineages. The *Drosophila* gene *Capa* encodes three neuropeptides: Capa-1, Capa-2, and Capa-3. Capa-1 and Capa-2 are related to the lepidopteran hormone cardioacceleratory peptide 2b, while Capa-3 is a novel member of the pheromone biosynthesis-activating neuropeptide/diapauses hormone/pyrokinin family. *Capa* is the first gene known to encode neuropeptides that act on renal fluid production through nitric oxide (Kean et al., 2002).

The Capa pre-propeptide is expressed in two subsets of cells in the VNC: two suboesophageal neurons, and 3 pairs of abdominal cells located in segments A2, A3 and A4 (this work;(Kean et al., 2002).We have focused our study on the abdominal Capa neurons (subsequently called ABCA cells). In this report we describe our work aimed at identifying the NB progenitor of the ABCA cells and their temporal gene window, and establish the groundwork for the genetic mechanisms involved in their specification.

In this manner, we first identified NB 5-3 as the most probable progenitor NB of the ABCA neurons. Second, we demonstrate that ABCA cells are generated within a *castor* (*cas*) temporal window, and that *cas* is critical for the specification of ABCAs. Third, we show that the ABCA sibling cell dies by apoptosis. Surprisingly, ABCA-like cells are generated in A5-A7 segments when cell death is blocked, suggesting that ABCA cells are generated in these segments, but die before expressing Capa. Finally, by means of a targeted genetic screen we have identified several putative components of the genetic combinatorial code specifying the ABCA neural fate. Mutations in 12 of the 42

genes analyzed show either reduced or excess number of ABCA cells. From these, *grunge* and *hedgehog* are absolutely required for ABCA specification and/or generation. We have extended this analysis to the suboesophageal Capa cells (SECA cells), and show that expression of the same terminal identity gene *Capa* can be achieved from different temporal window, using distinct genetic mechanism. This study provides the foundation for an attractive new paradigm in which to address questions related to neuronal specification.

2. RESULTS

2.1. Expression pattern of the Capa peptide in the VNC of *Drosophila melanogaster*

To examine the expression pattern of the *Drosophila* Capa neuropeptide in early development, we used a polyclonal antibody against the Capa pro-peptide (pro- Capa; henceforth called Capa antibody) (Kean et al., 2002). Capa expression is highly restricted in the VNC; it is expressed in 8 neurons, divided in two subsets of cells: the SECA cells comprise two neurons in the third suboesophageal segment (Fig.1A and S1; (Kean et al., 2002), and the ABCA cells consist of 6 abdominal cells, one in each of the A2-A4 hemineuromeres (Fig.1A and S1;(Kean et al., 2002). An additional pair of subesophageal cells in the second suboesophageal segment (SE2CAs; asterisk in Fig.1A, Fig. S1) shows an extremely weak Capa expression (asterisk in Fig. 1A), and thus was not further analyzed. Expression of the *Drosophila* Capa neuropeptide commences at early embryonic stage 17, where SECA cells are firstly detected (Fig. 1B). ABCA neurons appear immediately after, at the middle of stage 17 (Fig. 1C). This expression pattern is maintained, at least, until the third larval instar (Fig. 1D).

Both ABCA and SECA neurons express the bHLH protein Dimmed (Dimm), encoded by the neuropeptidergic master gene *dimm* (Gauthier and Hewes, 2006; Hamanaka et al., 2010; Hewes et al., 2003; Park et al., 2008) (white arrowhead in Fig. 1E-E´´;(Park et al., 2008). However, SE2CA cells do not express Dimm (black arrowhead in Fig. 1E-E´´; (Park et al., 2008). Previous reports suggested that the SECA cells also express FMRFa neuropeptide (Park et al., 2008). However, we did not find co-localization between FMRFa and Capa antibodies (Fig. 1F-F´´), demonstrating that SE2 (FMRFa) and SECA (Capa) neurons are different cells.

To summarize, Capa expression is highly restricted in the *Drosophila* VNC. Capa is expressed in two subsets of cells: two cells in the third suboesophageal segment (SECAs) and six cells in the A2-A4 segments (ABCAs).

[Figure.1]

2.2. Identification of the ABCA progenitor neuroblast (NB)

To date, none of the lineages that generate the Capaergic neurons have been identified. Therefore, we focused our attention in identifying the progenitor NB that gives rise to the ABCA neurons. Extensive work during the last two decades has provided a detailed map of most, if not all, of the 30 NBs generated in each hemisegment, providing a set of genetic markers that permit identification of the different NBs in the embryonic VNC, born between stages 8 to 11 (Bossing et al., 1996; Doe, 1992; Prokop and Technau, 1991; Schmid et al., 1999; Schmidt et al., 1997). Given that Capa expression is first detected late in embryonic development (stage 17), and we do not have other earlier markers to identify ABCA neurons, we performed co-expression analysis of Capa and NB markers at stage 18h After Egg Laying (AEL).

ABCA neurons express *gsb-lacZ* (a row 5, 6 and NB 7-1 marker) and *wingless-lacZ* (*wg-lacZ)* (a row 5 NB marker; Fig. 2A, B and I), but do not express *engrailed-GAL4* (*en-GAL4*) (a row 6 and 7 NB marker; Fig. 2 C and I). These results strongly suggest that ABCA neurons arise from row 5 NBs (Fig. 2I).

Because migration of neurons is limited within the VNC, and ABCA neurons show a medial position within row 5 (Fig. 2D), the medial NB5-1, 5-2, and 5-3 were the most likely progenitor NBs. However, we could not discard other NBs from row 5 as putative progenitors of ABCA cells, and therefore used additional markers to distinguish between different row 5 NBs. One of these markers is the *ladybird-early (K)-GAL4* (*lbe(K)-GAL4*) reporter construct, in which GAL4 expression is driven by a 2-kb fragment (called K) located 5-kb upstream of the *lbe* gene transcription start site. Unlike the *lbe* gene, which is expressed in subsets of cells of the NB 5-3 and 5-6 lineages, this transgenic marker is specifically expressed in NB5-6 and its progeny.(Baumgardt et al., 2009; De Graeve et al., 2004). We found that ABCA neurons do not express the NB5-6 specific transgenic marker *lbe(K)-GAL4*) (Fig. 2E), discarding NB5-6 as progenitor of

the ABCA neurons. On the contrary, ABCAs neurons express *unplugged-lacZ* (*unpg-lacZ*) (Fig. 2F), which is expressed by NB 5-3 and 5-5 (Fig. 2I). To distinguish between these two NBs, we use the *huckebein-lacZ* (*hkb-lacZ*) marker, which is expressed by NB5-5, but not by NB5-3. We did not find *hkb-lacZ* expression in ABCA cells (Fig. 2G). Therefore, both positional and labeling cues support the hypothesis that ABCA cells are generated by NB 5-3. Since the Ladybird-early marker is expressed by a subset of cells deriving from NB 5-3 (De Graeve et al., 2004), we asked if the ABCA cell belongs to that subset. Unfortunately, the ABCA cells do not express Ladybird-early (Fig. 2H).

To summarize, our findings strongly suggest that the ABCA neurons are generated by NB 5-3.

[Figure.2]

2.3. Identification of the temporal genes involved in the specification of the ABCA neurons

In order to identify the temporal window in which the ABCA cells are born, we examined Capa expression in mutants for each temporal gene: *kr*, *pdm*, *cas*, and *grh* (genotypes in legend of Fig. 3 and Experimental Procedures). Unfortunately, we were not able to study *hb* loss-of-function, since the death of *hb* mutants during early stages precluded the identification of the Capa cells at stage 17 (in spite of using hb^{P1} hb^{FB} mutants to remove only *hb* CNS expression (Isshiki et al., 2001); we also tried *elav/prospero-GAL4>UAS-HbRNAi*, even in combination with *UAS-DicerII*, but Hb antibody was never gone in those genetic backgrounds; data not shown).

ABCA cells were unaffected in all but *castor* (*cas*) mutants (Fig. 3A-E, I and J), where we observed a complete absence of ABCA neurons (Fig. 3D, I and J). This strongly suggests that ABCA cells are born during the *cas* temporal window. To further support this hypothesis, we analyzed Cas protein expression in the ABCA cells. In agreement with the loss-of-function results, Cas antibody labeled ABCA neurons (Fig. 3G).
To test whether *cas* may be sufficient to trigger ectopic Capa cells, we analyzed the effect of mis-expressing *cas* with the pan-neural driver *elav-Gal4*. We found a wild type phenotype (Fig. 3F, I and J). The same result was found when we mis-expressed Cas with the *prospero-Gal4* driver (data not shown). This result indicates that *cas* is

necessary, but not sufficient to specify the ABCA fate, and thus more factors must be involved in this specification. A requirement for *grh* in this process was ruled out, because Capa expression is completely normal in *grh* mutants (Fig.3E, I and J) and the Capa-positive cells do not express Grh (Fig. 3H). In summary, our data strongly suggest that the ABCA neurons are born during the Cas temporal window.

[Figure.3]

2.4. The role of Programmed Cell Death and Notch Pathway in ABCA specification

ABCA neurons are generated by NB 5-3 during the Cas temporal window. It is known that the Ganglion Mother Cell (GMC) progenitors divide only once to generate two invariant cells that will differentiate as neuron or glia cells, or undergo Programmed Cell Death (PCD) (reviewed in (Karcavich and Doe, 2005). In two out of the three peptidergic lineages that are best understood (FMRFa, Leucokinin (LK), and Corazonin (Crz)), two neurons expressing the same neuropeptide (LK or Crz) are generated from the GMC, but one of them dies by PCD in a Notch (N) dependent fashion (Benito-Sipos J., 2010; Karcavich and Doe, 2005). Therefore, we asked whether the ABCA neuron has an ABCA-sibling cell undergoing PCD. In such a case, we would see another ABCA cell in a scenario where PCD is blocked. In *Drosophila*, PCD is critically dependent upon a family of IAP inhibitors, the RHG-motif genes *reaper* (*rpr*), *grim* and *head involution defective* (*hid*) (Bergmann et al., 2003). Embryos homozygous for the chromosomal deletion *Df(3L)H99* (*H99*) lack these three genes and show an apparently complete absence of apoptosis (White et al., 1994).Thus, we examined Capa expression in a *H99* mutant background. We found two ABCA neurons per hemisegment (Fig. 4B, H and I), indicating that the ABCA sibling cell dies by apoptosis, but produces an ABCA neuron if PCD is inhibited. Surprisingly, we found two ABCA neurons not only in the A2-A4 hemisegments, but also in the A5-A7 hemineuromeres (Fig. 4B and I). This result indicates that the NB 5-3 also produces a GMC capable of generating the ABCA cell and its sibling in the A5-A7 hemisegments, but either the GMC or the two postmitotic neurons undergo cell death specifically in those segments. Unexpectedly, we obtained a different result by the over-expression of the baculovirus effector caspase inhibitor *p35* (*elav-Gal4>UAS-p35*) (Hay et al., 1994). Instead of the two ABCA cells per hemisegment (from A2 toA7) we only found one ABCA cell per hemisegment, in

the six abdominal segments A2 to A7 (Fig. 4C and I). Surprisingly, when a *cas-Gal4* driver was used to express p35 the result was different, and two ABCA neurons were frequently detected in A2-A7 hemineuromeres (Fig. 4D and I). The different expression timing of the two drivers could explain these results, which suggest that the death of the two ABCA sibling cells occur at different times (see discussion).

It has been reported in several lineages that N signaling between the two GMC daughter cells is required for them to assume different cell fates (Lundell et al., 2003; Schuldt and Brand, 1999; Skeath and Doe, 1998; Spana and Doe, 1996). It has also been shown that N signaling controls PCD in post-mitotic cells in a lineage specific manner (Benito-Sipos J., 2010; Karcavich and Doe, 2005; Lundell et al., 2003; Novotny et al., 2002). Therefore, we studied the role of N signaling in the specification of the ABCA cells by examining Capa expression in N pathway mutants. Unfortunately, the death of *sanpodo* and *mastermind* mutants during late stage 16 precluded the identification of the ABCA cells. Thus, to study the effect of deficient N signaling, we analyzed mis-expression of *numb* (*Cas-Gal4>UAS-numb*). The membrane-associated Numb protein antagonizes N signaling to specify sibling neuron cell fates (Spana and Doe, 1996). In this situation, we observed a tendency to produce duplications (Fig. 4E, H and I). We next analyzed the effect of N gain of function, by expressing a constitutively active form of N (*cas-Gal4>UAS-*N^{intra}) (Rebay et al., 1993; Struhl et al., 1993). In agreement with the results from lack of N signaling, we observed the opposite phenotype, a total absence of ABCA cells (Fig. 4F, H and I), and we obtained similar results when N^{intra} was expressed with the *elav-Gal4* driver (data not shown). According with these set of data, ABCA cells seem to be "Noch OFF" neurons. In order to verify this hypothesis we simulated the over-activation of the N pathway using *numb* mutants. We found a complete absence of ABCA neurons also in *numb* mutants (Fig. 4G, H and I). Therefore, our results demonstrate that N signaling is indeed involved in ABCA specification, and suggest that the absence of N signaling is necessary to prevent the death of these neurons..

In summary, the ABCA sibling cell dies by apoptosis but displays an ABCA phenotype if PCD is inhibited. On the other hand, the NB 5-3 also produces a GMC-generating ABCA cell and its sibling in the A5-A7 hemisegments, but the GMC or the neurons die before Capa expression commences. In addition, we have found that correct specifications of the ABCA cells requires silencing of Notch signaling .

[Figure.4]

2.5. Identification of genes required for the specification of the ABCA neuronal fate

It is becoming increasingly clear that neuronal fate specification is a multistep process involving combinatorial gene expression codes specifying neuronal properties (Allan et al., 2005; Baumgardt et al., 2007; Certel and Thor, 2004; Garces and Thor, 2006). In order to identify genes involved in the specification of the ABCA fate, we analyzed the expression of Capa in embryos mutant for genes known to be expressed in the CNS (Brody et al., 2002). We identified a number of mutants in which the pattern of the ABCA neurons was not altered: *apterous (ap), atonal (ato), beadex (bx), crooked legs* (*crol*)*, dachshund (dac), dimmed (dimm), defective proventriculus* (*dve*)*, eagle(eg), elbow/noc ocelli (el/noc), empty spiracles (ems), eygone (eyg), hearthless (htl), jumeaux (jumu), ken and barbie (ken), knirps(kni),lim3, nab, panier(pnr), schnurri (shn), scribble (scrib), squeeze (sqz), shuttle craft (stc), seven up* (*svp), rhea, target of Poxn (tap), tonochaetae (ton), tailup* (*tup*)*, vestigial* (*vg*)*, vein (vn)* (Table 1); others in which the number of the ABCAs was significantly increased: *chip, osa, rotund (rn), fork head (fkh)* and *klumpfuss (klu)* (Table 1); and others where the number of the ABCA cells was significantly decreased: *collier (col), daughterless (da), zinc finger homeodomain 2 (zhf2), fushi tarazu (ftz) and grain* (Table 1).

Among the genes in which we found an increase of the ABCA neurons, the strongest phenotype was found in *klu* and *rn* mutants (Fig 5B, C, J and K; Table 1). On the other hand, among the genes that produce a decrease of the ABCA cells, the most striking phenotypes were found in: *zfh2*, *ftz* and *grain* (Fig. 5D-F, J and K; Table 1). Additionally, we found that *Grunge* and *hedgehog* genes are crucial for the specification or generation/survival of ABCA cells, because mutations in any of these genes lead to an almost complete absence of ABCA cells (Fig. 5G, H and J; Table 1).

Since the ABCA cell seem to innervate some structure similar to the dorsal neurohemal organ (DNH; Fig. S2; (Gorczyca et al., 1994; Nassel et al., 1988; Santos et al., 2006), and various capa encoded neuropeptides have been identified in the adult peptidome of the CNS-associated abdominal neurohemal organs (Predel et al., 2004) we studied the possibility that a retrograde signal is involved in ABCA specification. In this manner,

we interfered with retrograde axonal transport by expressing a dominant-negative version of the P150/Glued dynactin motor complex component (*UAS-Glued* ^{DN}) with the *elavGAL4* pan-neural driver (Allen et al., 1999). In *elavGAL4>UAS-Glued*^{DN} 18 h AEL embryos we observed a wild type expression of the ABCA cells (Fig. 5I, J and K). Hence, retrograde signaling is not necessary for proper ABCA specification.

To summarize, we found that the number of ABCA cells is increased in *klu* and *rn* mutants, and decreased in the *zfh2*, *ftz* and *grain* mutants. Likewise, the *grunge* and *hedgehog* genes are crucial for the ABCA specification.

[Figure.5]

2.6. The SECA cells are born from the NB5-3 during a mixed *pdm*/*cas* temporal window, and they use a different genetic combinatorial code than the ABCA cells for their specification

Finally, we wanted to address whether the genetic requirements necessary to specify the ABCA cells are shared for specification of the SECA neuronal fate. First, we identified the progenitor NB that gives rise to the SECA neuron. SECA neurons express Ladybird-early marker but not *lbe(K)-GAL4* (Fig. 6A and B). Therefore, the SECA neurons are generated by NB5-3 (De Graeve et al., 2004). Moreover, the rest of the NB markers studied are consistent with this interpretation (data not shown). Second, we examined the pattern of SECA cells in mutants for each temporal gene. SECA neurons were unaffected in all these mutants except for *pdm* and *cas* mutants (Fig. 6D, E, M and N). In addition, the SECA cells expressed the Cas protein (Fig. S3). Unfortunately, we could not perform studies of co-localization between Capa and Pdm because both antibodies were generated in rabbit. Thus, our findings suggest that SECA cells are born during a mixed *pdm*/*cas* temporal window. Next, we studied SECA expression pattern in all mutants identified in the specification of the ABCA cells. We found that only *grunge* and *hedgehog* are involved in SECA specification, but the reduction in the number of SECA cells in *hh* and *grunge* mutants is highly variable, and not as dramatic as that observed for ABCA cells (Fig.6F-N).

In summary, the SECA cells are generated by the NB 5-3 during a *pdm*/*cas* temporal window, and for its proper specification they only share the action of the *grunge* and *hedgehog* genes with the ABCA cells.

[Figure.6]

3. DISCUSSION

Understanding how neuronal diversity is achieved remains one of the central challenges in neurobiology. Tremendous effort by many laboratories over the past two decades has led to elucidate the molecular genetic mechanisms that control nervous system development in *Drosophila*. The impact of these studies on the field of vertebrate developmental neurobiology has been profound. Nevertheless, several important issues still remain to be resolved. How is the spatial-specific expression of the large numbers of neurotransmitters/neuropeptides and their receptors controlled? There are very few examples where this question has been addressed, and it remains unknown if the genes and the mechanisms found are shared by other similar systems. Additionally, there are too few *Drosophila* neurons/glia where the progenitor NB and/or the temporal genes that give rise to those cells have been identified .In this report, we describe the lineage origin of the Capaergic neurons in the VNC, and present the results of a genetic screen that provides the framework for analyzing the mechanisms involved in their specification. Some features of this peptidergic system make them specially suited for addressing essential issues in neurodevelopment such as the segmental regulation of cell identity, or the role of programmed cell death in cell fate determination.

3.1. ABCAs cells are generated in the Castor temporal window by the NB 5-3

In the *Drosophila* VNC, each hemisegment is formed by about 400 postmitotic cells derived from an invariant set of 30 NBs (reviewed in (Karcavich and Doe, 2005). Most, if not all, postmitotic cells within each hemisegment are unique. This cell individuality is achieved by mechanisms operating at the NB, GMC, and neuron/glia cell. Therefore, a complete understanding of the regulatory networks acting to specify a particular fate demands knowledge of the NB and GMC progenitor cells. Our findings strongly suggest that the capaergic abdominal ABCA neuron arises from NB 5-3. This conclusion is based on the expression in ABCA cells of *gsb*, *wg* and *unpg*, and the absence of the markers *lbe(K)* and *hkb*. However, even though *gsb* expression is known

to be maintained specifically in the lineage of rows 5 and 6 NBs (Buenzow and Holmgren, 1995), whether expression of the other genetic markers used to identify NBs at stage 11 changes late in embryogenesis remains unanswered. Nonetheless, the specific combination of NB markers found in ABCA cells and their position in the hemineuromere are consistent with their birth from NB5-3. Previous accounts showed that this NB gives rise to a lineage of 9 to 15 cells (Bossing et al., 1996). Additionally, observations derived from studies in which PCD was blocked (Rogulja-Ortmann et al., 2007) showed that NB 5-3 can potentially produce a large lineage (ranging from 19 to 27 cells), suggesting that it could generate 13 or 14 GMCs. The lack of a NB 5-3 specific-lineage marker prevented us from resolving its complete lineage, and thus determining the birth order of the ABCA cell.

Recent findings on the NB 5-6 and NB 5-5 demonstrate that *cas* and *grh* act together as critical temporal genes to specify peptidergic cell fates at the end of these lineages (Baumgardt et al., 2009; Benito-Sipos J., 2010). We have observed that *cas* mutants lack ABCA cells and that Cas is expressed in these cells, while the normal pattern of ABCA cells is found in *grh* mutants, and Grh is not present in ABCA neurons. These data strongly support the birth of ABCA cells in a *cas*-only temporal window. This is different from the suboesophageal Capaergic SECA cells, which while also arising from NB5-3, show a reduction in cell number in both *pdm* and *cas* mutants, demonstrating birth at a mixed *pdm/cas* temporal window. Previous studies in other lineages have shown that when a temporal gene is mis-expressed, all progeny cells posterior to that temporal window can be transformed to the specific fate born at that particular temporal window. However, *cas* mis-expression failed at inducing ectopic ABCA cells, suggesting that *cas* in necessary but not sufficient to specify the ABCA fate.

3.2. Role of Notch signaling and programmed cell death in ABCA cell fate

Programmed cell death (PCD) is a basic process in normal development. Our results suggest that the ABCA and its sibling are equivalent cells committed to achieve the ABCA fate. First, we have shown that the ABCA sibling cell dies by apoptosis, but produces an ABCA-like capaergic neuron if PCD is inhibited. Second, when PCD is blocked, NB 5-3 also produces a GMC generating two ABCA-like capaergic cells in the A5 to A7 segments. These data indicate that a segment-specific mechanism prevents death of the ABCA cells in A2-A4 neuromeres. Segment specific cell death has been

previously reported for the NB5-3 lineage (Rogulja-Ortmann et al., 2007), and detailed studies on segment-specific apoptosis of other lineages have shown that this process is under homeotic control (Miguel-Aliaga and Thor, 2004). In addition, our results show a different timing in the PCD undergone by the ABCA sibling and the ABCA cells born in A5-A7. This interpretation is based on the differential effect of p35 expression when *cas-Gal4* or *elav-Gal4* drivers were used. Although *elav-Gal4* is transiently express in NBs and GMCs (Berger et al., 2007), robust and maintained driver expression commences in differentiating neurons. On the other hand, *cas* expression starts in the NB and is maintained in the GMC and neuronal progeny. Therefore, the finding that death of the ABCA sibling cell can be prevented by directing p35 with *cas-Gal4*, but not with *elav-Gal4*, suggests that the death of the ABCA sibling occurs earlier in development than the death undergone by the ABCA cells in A5-A7 segments.

In the ABLK/LK peptidergic fate (derived from the NB 5-5), activation of Notch (N) signaling in the peptidergic cell prevents its death, while its sibling, N^{OFF} cell undergoes apoptosis (Benito-Sipos J., 2010). On the contrary, in the EW3/Crz peptidergic fate (derived from the NB 7-3), silencing of N signaling is essential for the neuron survival, and therefore for it proper specification (Karcavich and Doe, 2005). Our results are in accordance with the last scenario, in which the ABCA cell is N^{OFF}, and it sibling, which undergoes apoptosis, is N^{ON}. Therefore, Notch signaling must be switch off for the proper specification of the ABCA neuron.

3.3 The targeted genetic screen is an efficient method to approach the study of the specification of neural fates

To search for genes involved in specification of the ABCA neural fate, we screened a reduced set of mutants on genes that are expressed in the embryonic CNS at stage 11, a time at which distinctly defined sublineages are being generated from all active NBs (Brody et al., 2002). Even though this method will certainly overlook important genes, our results reveal that it is in fact a very effective way to find genes involved in specification of a particular neural fate. Indeed, the ratio of success has been very satisfactory: 33,3% of the genes analyzed display a significant phenotype. Moreover, the set of identified genes could be further expanded by, for example, searching in interactome databases, and performing the subsequent screen on those putative interactors.

It is assumed that the specification of a concrete cellular fate requires the combination of several transcription factors, namely a genetic combinatorial code. Recently, a detailed combinatorial code has been reported for three neuropeptidergic fates: ap4/FMRFa, ap1/Nplp1 and ABLK/Lk (Baumgardt et al., 2009; Baumgardt et al., 2007; Benito-Sipos J., 2010). However, very little is known about the specification of the rest of the 30 peptidergical fates (Park et al., 2008). We have identified several genes involved in the specification of the ABCA fate, which fit into three categories. First, we have found genes whose loss-of-function produces a relevant increase of the number of ABCA cells. Most remarkable are the *klu* and *rn* phenotypes, which consist of duplications of the ABCA cells. These phenotypes suggest that these two transcription factors repress the ABCA fate in other neural cells (or/and NBs/GMCs). Interestingly, the normal phenotype of *nab* mutants indicates that, contrary to its mode of action in the wing, Rn does not work with the transcription cofactor Nab in this context (Table 1; (Terriente Felix et al., 2007).

Second, we have found genes whose loss-of-function produces a significant decrease of the number of ABCA neurons. In this category, the *zhf2*, *ftz* and *grain* phenotypes stand out. The effects of mutations on *ftz* are in agreement with its early role in segmentation: *ftz* is a pair-rule segmentation gene that defines even-numbered parasegments in the early embryo (Wakimoto et al., 1984), and we find absence of ABCA cells in the A3 segment (Fig.5E) in *ftz* mutants. However, *zfh2* and *grain* seem to be part of the specific combinatorial code of the ABCA cells. The *Drosophila* GATA transcription factor Grain has been reported to be involved in the specification of other cell fates, such as the aCC motoneuron fate (Garces and Thor, 2006). Based on its expression, the zinc finger homeodomain protein *zfh2* has been proposed to mediate specification of the serotoninergic fate (Lundell and Hirsh, 1992), but this has not been further demonstrated. Interestingly, during wing formation, *zfh2* is required for establishing proximo-distal domains in the wing disc, and it does so partly by repressing gene activation by Rn (Terriente Felix et al., 2007). The opposite phenotypes that we have observed in *rn* and *zfh2* mutants suggest that similar interactions occur during ABCA specification. We are currently performing analyses aimed to test this hypothesis.

Third, we have found two genes whose loss-of-function abolishes the ABCA fate: *grunge* and *hh*. *grunge* encodes a member of the Atrophin family of transcriptional co-

repressors that plays multiple roles during *Drosophila* development. Taken together, studies from *C. elegans* to mammals suggest that Atrophin proteins function as transcriptional co-repressors that shuttle between nucleus and cytoplasm to transduce extracellular signals, and that they are part of a complex gene regulatory network that governs cell fate in various developmental contexts (Shen and Peterson, 2009). Similarly, Hh is an extracellular signaling molecule essential for the proper patterning and development of tissues in metazoan organisms (reviewed in (Wilson and Chuang).. It is noteworthy that two genes implicated in extracellular signaling pathways, *grunge* and *hh*, are absolutely required for ABCA fate. Further studies will be needed to identify at which step/s they exert their actions, and to unravel possible interactions between them and with other players of the combinatorial code for ABCA specification.

4. EXPERIMENTAL PROCEDURES

4.1. Fly Stocks

Fly stocks were raised and crosses were performed at 25°C on standard medium. The following fly mutant alleles were used: *ap* ^{p44} (provided by S. Thor) , *ato* ^{1} , *bx* ^{1}, *cas*$^{\Delta 1}$, *chip*$^{e5.5}$, *col* ^{3} (provided by S. Thor), *crol*^{04418}, *da* ^{1}, *dac* ^{9} (provided by S. Thor), *dimmed*^{P} (provided by S. Thor)^{1}, *dve*$^{01D01W\text{-}L186}$ (provided by F.J.Díaz-Benjumea), *eg*^{2}, *el*^{331}*noc* $^{\Delta 64}$, *ems* ^{1}, *eyg* ^{2} , *fkh* ^{6}, *ftz*^{10}, *grn* ^{h10}, *Df(2R)Pcl7B (*referred to as *grh*^{Df}; provided by S. Thor),*grh*^{IM} (provided by S. Thor) , *gug*^{03928} , *hh* ^{ts2}, *hb*^{P1} *hb*^{FB}, a genetic combination that removes Hb CNS expression (Hulskamp et al., 1994; Isshiki et al., 2001) (provided by C.Doe) , *htl* ^{AB42} , *jumu* ^{11678}, *jumu* ^{11683}, *ken* ^{02970} , *klu* ^{212IR51C} , *kni*$^{RI\text{-}1}$, *kr*^{1} *kr*^{CD} to remove Kr CNS expression (Isshiki et al., 2001; Romani et al., 1996) (provided by C.Doe) , *lim3* ^{2}, *nab* ^{R52} (provided by F.J.Díaz-Benjumea), *osa*^{2}, *Df(2L)ED773* (referred to as *pdm* $^{(Df(2L)ED773)}$) (Grosskortenhaus et al., 2006)(provided by F.J.Diaz-Benjumea)*; ,pnr* ^{1} , *rn*$^{roe\text{-}1}$, *spdo*^{G104} (provided by S. Thor) ,*schurri*^{1} , *sqz* ^{ie} (provided by S. Thor), *stc*^{05441}, *svp* ^{1} (provided by S. Thor), *talin*^{1}, *tap* ^{01658}, *tll* ^{l49}, *ton*^{hdl}, *tup* ^{1}, *vg* ^{nw}, *vn* ^{C221}, *zfh2*^{MS209R20} (provided by I. Rodríguez). *LacZ* lines: *eve-lacZ*, *gsb*^{01155}*-lacZ*, *hkb*^{5953}*-lacZ*, *klu*^{09036}*-lacZ*, *lbe*^{K}*-lacZ* (this transgenic line contains a 2-kb genomic fragment of the regulatory region of *lbe* driving *lacZ*, and reproduces the pattern of expression of *lbe* (Baumgardt et al., 2009; De Graeve et al., 2004) (provided by S. Thor), *mir-lacZ* (provided by S. Campuzano), *unpg*^{r37}*-lacZ*, *wg-lacZ* (provided by F.J.Diaz-Benjumea). *Gal4* lines: *cas-Gal4* (provided by S. Thor), *elav-Gal4*, *prospero-*

Gal4 (provided by S. Thor), *lbe(K)-Gal4; UAS- nmEGFP* (provided by S. Thor). *UAS* lines: *UAS-dicer* on the II and III chromosomes (provided by M. Calleja); *UAS-cas* (provided by S. Thor), *UAS-Nintra* (provided by A. Baonza), *UAS-p35* (provided by M. Calleja), *UAS-hbRNAi* (V.D.R.C. # 44892), *UAS-Glued^{DN}*. Mutants were kept over *CyO, Act-GFP*; *CyO, Dfd-EYFP*; *TM3, Ser, Act-GFP*; *CyO, twi-Gal4, UAS-GFP*; *TM3, Sb, Ser, twi-Gal4, UAS-GFP*; or *TM6, Sb, Tb, Dfd-EYFP* balancer chromosomes. As wild type, *orizo 2* was often used. Unless otherwise stated, flies were obtained from the Bloomington Drosophila Stock Center.

4.2. Immunohistochemistry

The antibodies used were: Rabbit α-Capa (1:1000) (Kean et al., 2002); guinea pig α-Dimm (1:1,000) , chicken α-proFMRFa (1:1000) , rat α-Grainyhead (1:1,000) (all of them provided by S. Thor)(Baumgardt et al., 2009); Rabbit α-Cas (1:250) (Kambadur et al., 1998) (provided by S. Thor); Rabbit α-Ladybird-early (provided by K.Jagla) (De Graeve et al., 2004);Mouse α-βGal (JIE7) (1:200) (from Developmental Studies Hybridoma Bank, Iowa City, IA, US). All polyclonal sera were pre-absorbed against pools of early embryos. Secondary antibodies were conjugated with FITC, Rhodamine-RedX or Cy5, and used at 1:500 (Jackson ImmunoResearch, PA, US). Embryos were dissected in PBS, fixed for 25 minutes in 4% PFA, blocked and processed with antibodies in PBS with 0.2% Triton-X100 and 4% donkey serum. Slides were mounted with Vectashield (Vector, Burlingame, CA, US). In all cases wild-type and mutant embryos were stained and analyzed on the same slide.

4.3. 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 facilitate for color-blind readers or to facilitate the understanding of the paper.

4.4. 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.

ACKNOWLEDGMENTS

We are grateful to S. Thor, JA. Veenstra , A. Hidalgo, K. Jagla , C. Doe, W. Chia, M. Calleja, A. Baonza, I. Rodríguez, F. J. Diaz-Benjumea, S. Campuzano and the Developmental Studies Hybridoma Bank and the Bloomington Stock Center for sharing antibodies and fly lines. We thank M. Baumgardt, F. Martín and S. Casas for comments on the manuscript. We also want to thank to Carlos Sánchez,Verónica Labrador and Angeles Muñoz for their technical assistance in confocal microscopy. This work was supported by a grant from the Spanish Ministerio de Ciencia e Innovación (BFU-2008-04683-C02-02 to L.T.).

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FIGURE LEGENDS

Figure 1.

Expression pattern of Capa in the *Drosophila* VNC.

(A) Staining for Capa in 18-h AEL VNC. The six ABCA neurons reside medially in the abdominal hemisegments, one in each of the A2-A4 hemineuromeres. The SECA neurons reside in the third suboesophageal segment. Another pair of suboesophageal cells are found in the second suboesophageal segment (asterisk), showing an extremely weak Capa expression.

(B) Staining for Capa in early stage 17 VNC. SECA cells are first detected at early embryonic stage 17.

(C) Staining for Capa in middle stage 17 VNC. ABCA cells are first detected at the middle stage 17.

(D) Staining for Capa in the third larval instar. The embryonic expression pattern is maintained, at least, until LIII.

(E-E´´) Overlap of Capa (green) and Dimm (magenta). Both the ABCA and SECA neurons (white arrowheads), but not SE2CA (black arrowhead), express Dimm. See text for details.

(F-F´´) Overlap of Capa (green) and FMRFa (magenta). Both neuropeptides are expressed in an independent fashion, indicating that SE2s (FMRFa) and SECAs (CAPA) neurons are different cells.

Anterior is up in all images.

Figure 2.

ABCA neurons seem to be generated by the NB 5-3 A

(A-H) Overlap of Capa (green) and different progenitor NB markers (magenta): *gsb-lacZ* (A, D), *wg-lacZ* (B), *en-lacZ* (C), *lbe (K)-Gal4* (E), *unpg-lacZ* (F), *hkb-lacZ* (G), Lbe (H).

(A-H) ABCA cells overlap with *gsb*, *wg*, *unpg* but not with *en*, *hkb*, Lbe and *lbe(K).*

(D) ABCAs neurons show a medial position within row 5 (ML=Midline; M=Medial; L=Lateral ; A= Anterior; P= Posterior).

(I) Cartoon summarizing the NB markers.

See genotypes above. Anterior is up in all images.

Figure 3.

ABCA neurons are specified in a Cas temporal window.

(A) ABCA/Capa expression in control; (B and I) kr^{1} kr^{CD}; (C and I) $pdm^{(Df(2L)ED773)}$; (D and I) $cas^{\Delta 1}/cas^{\Delta 1}$; (E and I) grh^{IM}/grh^{Df}; (F and I) *elav-Gal4>UAS-cas*.

(G) Overlap of ABCA/Capa (green) and Castor (magenta). Castor is expressed by ABCAs cells.

(H) Overlap of ABCA/Capa (green) and Grainyhead (magenta). Grainyhead is not expressed by ABCAs cells.

(I) Quantification of observed phenotypes (n≥10 VNCs in all genotypes). The asterisk (*) denotes significant difference compared to control (Student t-test, p ≤0.001).

(J) Cartoons summarizing the observed phenotypes.

See genotypes above. Anterior is up in all images.

Figure 4.

Study of the role of Programmed Cell Death and Notch Pathway in ABCA specification

Analysis of the ABCA neurons in different mutant background:

(A and H) Control; (B and H) *DfH99/DfH99*; (C and H) *elav-Gal4>UAS-p35*; (D and H) *cas-Gal4>UAS-p35*; (E and H) *cas-Gal4>UAS-numb*; (F and H) *cas-Gal4>UAS-*N^{intra}; (G and H) $numb^{1}/numb^{1}$

(H) Quantification of observed phenotypes (n≥10 VNCs in all genotypes). The asterisk (*) denotes significant difference compared to control (Student t-test, p ≤0.001). (†) denotes significant difference compared to control (Student t-test, p ≤0.05)

(I) Cartoons summarizing the observed phenotypes.

See genotypes above. Anterior is up in all images.

Figure 5.

Genes involved in the ABCA specification

Analysis of the ABCA neurons in different mutant background:

(A) Control; (B and K) $klu^{212IR51C}/klu^{212IR51C}$; (C and K) rn^{1}/rn^{1}; (D and K) $zfh2^{MS209R20}/zfh2^{MS209R20}$; (E and K) ftz^{10}/ftz^{10}; (F and K) $grain^{h10}/grain^{h10}$; (G and K) $Grunge^{03928}/Grunge^{03928}$, (H and K) hh^{ts2}/hh^{ts2} and (I and K) *elav-Gal4>UAS-*$glued^{DN}$.

(B and C) The ABCA cell number is increased in *klu* and *rn* mutants.

(D-F) The ABCA cell number is decreased in *zfh2*, *ftz* and *grain* mutants.

(G and H) The ABCA cell number is dramatically reduced or totally absent in *Grunge* and *hh* mutants.

(I) Retrograde signaling is not necessary for proper ABCA specification.

Anterior is up in all images.

(J) Cartoons summarizing the observed phenotypes.

(K) Quantification of observed phenotypes (see Table 1 for more details). The asterisk (*) denotes that the increase or decrease of the ABCA cell number is significant (Student t-test, $p \leq 0.001$). See genotypes above.

Figure 6.

Analysis of the SECA cell specification

(A and B) Overlap of Capa (green) and two progenitor NB markers (magenta):

(A) Ladybird-early antibody (Lbe); (B) *lbe (K)-Gal4>UAS-GFP*.

Capa overlaps with Lbe antibody, but not with *lbe (K)-Gal4.*

(C-N) Analysis of the SECA neurons in different mutant background:

(C) Control; (D) $pdm^{(Df(2L)ED773)}$; (E) $cas^{\Delta 1}/\ cas^{\Delta 1}$; (F) $klu^{212IR51C}/\ klu^{212IR51C}$; (G) $rn^{roe\text{-}1}/\ rn^{roe\text{-}1}$; (H) $zfh2^{MS209R20}/zfh2^{MS209R20}$; (I) $ftz^{10}/\ ftz^{10}$; (J) $grain^{h10}/\ grain^{h10}$; (K) $Grunge^{03928}/\ Grunge^{03928}$ and (L) $hh^{ts2}/\ hh^{ts2}$.

(D and E) The ABCA cells are absent in *pdm* and *cas* mutants.

(K and L) The ABCA cell number is dramatically reduced in *Grunge* and *hh* mutants.

Anterior is up in all images.

(M) Cartoons summarizing the observed phenotypes.

(N) Quantification of observed phenotypes ($n \geq 10$ VNCs in all genotypes). The asterisk (*) denotes that the increase or decrease of the ABCA cells number is significant (Student t-test, $p \leq 0.001$). See genotypes above.

Figure S1

SECA cells reside in the third suboesophageal segment and ABCA cells are found in each of the abdominal A2-A4 hemineuromeres

Overlap of Capa (green) and *gsb-lacZ* (magenta)

Figure S2

ABCA cells innervate some structure similar to the dorsal neurohemal organ

White arrowheads point at the ABCA cells. Black arrowheads point the DNH-like structure. The arrow points to the axon of the ABCA cell innervating this structure.

Figure S3

SECA cells are labeled with Cas antibody

Overlap of Capa (green) and Cas (magenta)

Table

TABLE 1

Genotype	**χ**	**S.D.**	**Nº**
Wild type	3.10	0.18	>100
ap ^{p44}	2,92	0,13	14
atonal ^{1}	3	0	12
bx ^{1}	3,05	0,19	19
chip$^{e5.5}$	3,25	0,38	16
collier 3	2,86	0,24	14
crol^{04418}	3	0	8
da ^{1}	2,78	0,34	14
dac ^{9}	3	0	9
dimmed^{P1}	3	0,14	14
dve$^{01D01W\text{-}L186}$	3	0	4
eagle^{2}	3	0	14
el^{331}*noc* $^{\Delta 64}$	2,85	0,34	20
ems ^{1}	3,07	0,21	26
eygon ^{2}	3,05	0,09	20
fkh ^{6}	3,21	0,37	14
ftz$^{\mathbf{10}}$	**1,77**	**0,36**	**10**
grain $^{\mathbf{h10}}$	**2,63**	**0,46**	**19**
Grunge$^{\mathbf{03928}}$	**0**	**0**	**13**
hedhog $^{\mathbf{ts2}}$	**0,54**	**0,49**	**12**
htl ^{AB42}	3	0,11	17
jumu ^{11678}	3	0	10
jumu ^{11683}	3,12	0,21	16
ken ^{02970}	3	0	14
klu $^{\mathbf{212IR51C}}$	**4,84**	**0,95**	**26**
knirps $^{RI\text{-}1}$	3	0	20
lim3 ^{2}	3	0	11
nab ^{R52}	3	0	12
osa^{2}	3,25	0,37	12
pnr ^{1}	3,10	0,29	19
rotund$^{\mathbf{roe\text{-}1}}$	**3,56**	**0,63**	**16**
schurri^{1}	3	0	8
sqz ^{ie}	3	0	12
stc^{05441}	3	0,22	9
svp ^{1}	2,85	0,21	16
talin^{1}	3	0	15
tap ^{01658}	2,88	0,20	9
tll ^{l49}	3,07	0,14	14
ton^{hdl}	3,11	0,29	28
tup ^{1}	3,08	0,15	12
vg ^{nw}	2,8	0,27	12
vn ^{C221}	2,94	0,11	17
zfh2 $^{\mathbf{MS209R20}}$	**2,11**	**1,09**	**26**

(χ): Average number of ABCAs per hemiganglion; S.D: Standard deviation ;(Nº): number of hemiganglia scored.

Figure 1

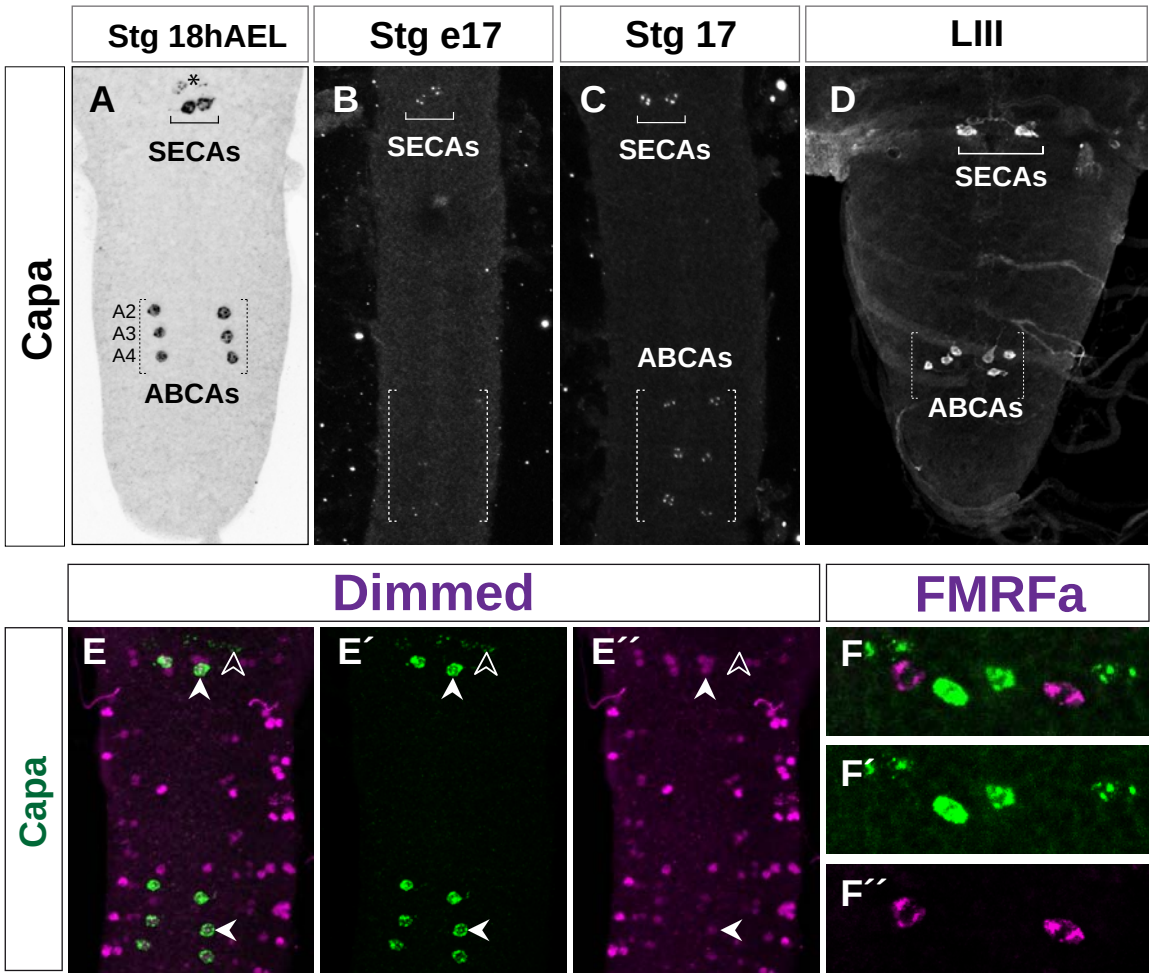
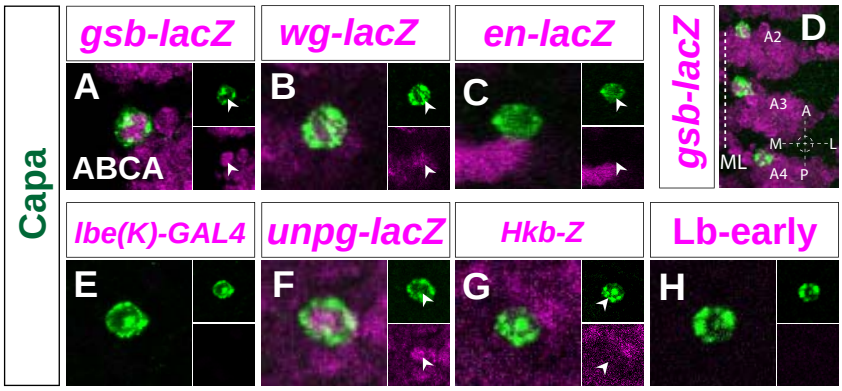

Stg 18hAEL
Stg e17
Stg 17
LIII
Capa
A
SECAs
A2
A3
A4
ABCAs
B
SECAs
C
SECAs
ABCAs
D
SECAs
ABCAs
Dimmed
FMRFa
Capa
E
E´
E´´
F
F´
F´´

Figure 2


Capa
gsb-lacZ
wg-lacZ
en-lacZ
gsb-lacZ
A
ABCA
B
C
D
A2
A3
A
M
L
ML
A4
P
lbe(K)-GAL4
unpg-lacZ
Hkb-Z
Lb-early
E
F
G
H

I

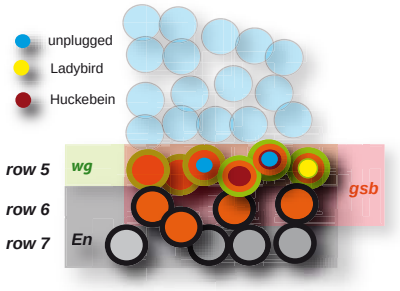

unplugged
Ladybird
Huckebein
row 5
row 6
row 7
wg
En
gsb

Figure 3

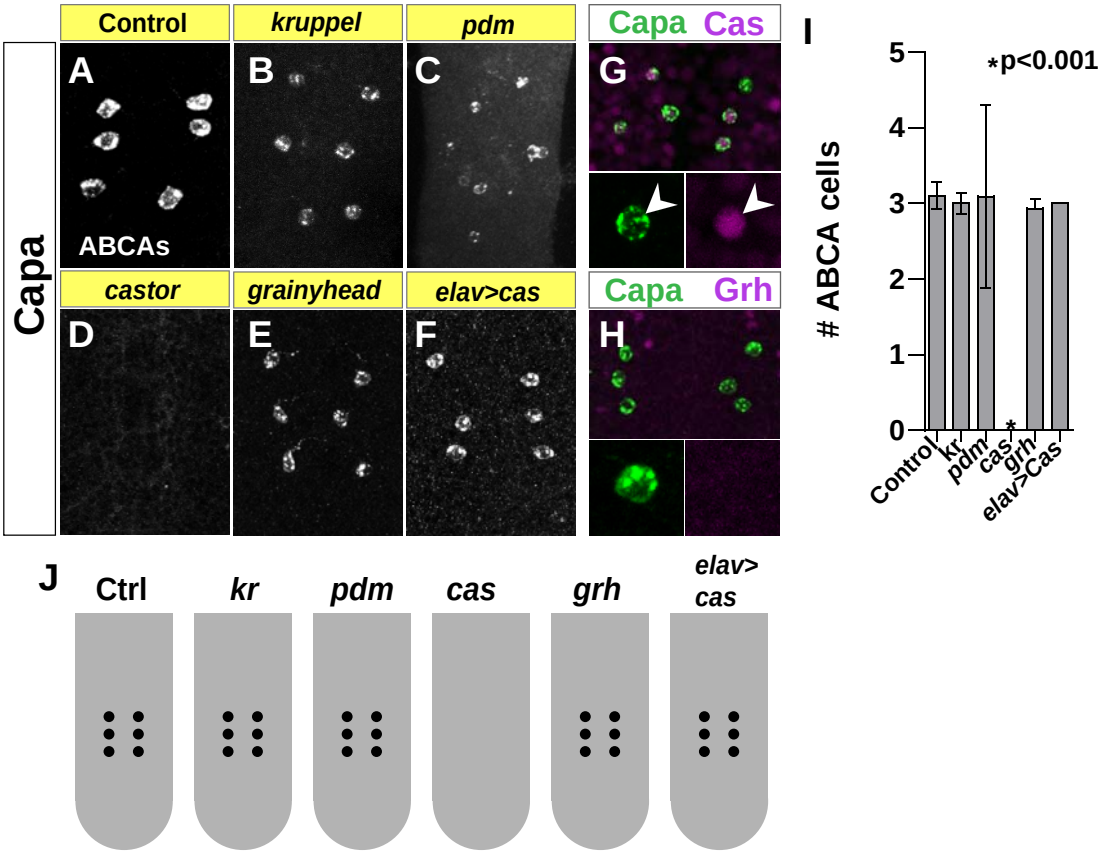

Control
kruppel
pdm
Capa Cas
Capa
A
B
C
G
ABCAs
castor
grainyhead
elav>cas
Capa Grh
D
E
F
H
I
ABCA cells
*p<0.001
5
4
3
2
1
0
Control
kr
pdm
cas
grh
elav>Cas
J
Ctrl
kr
pdm
cas
grh
elav> cas

Figure 4

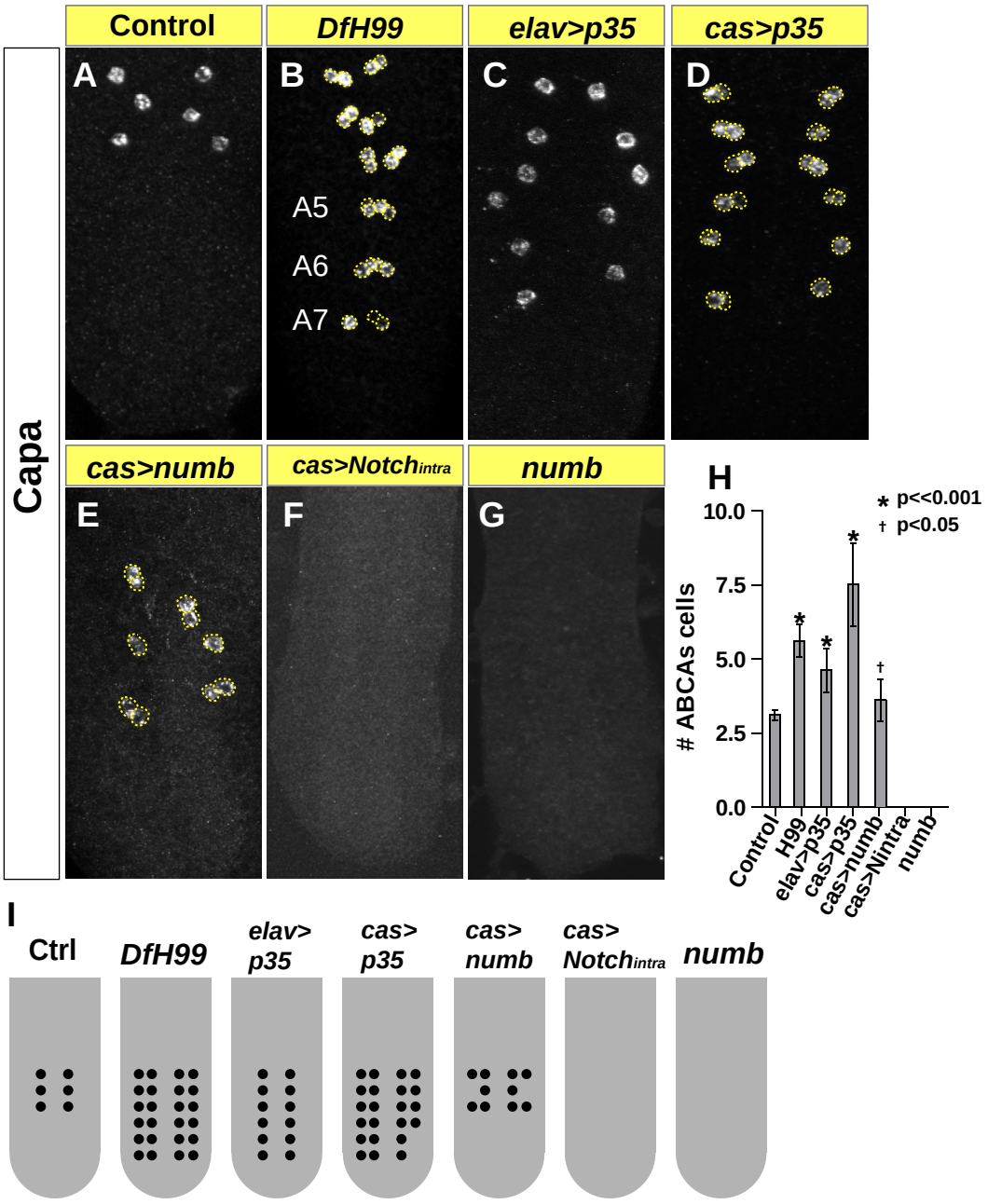
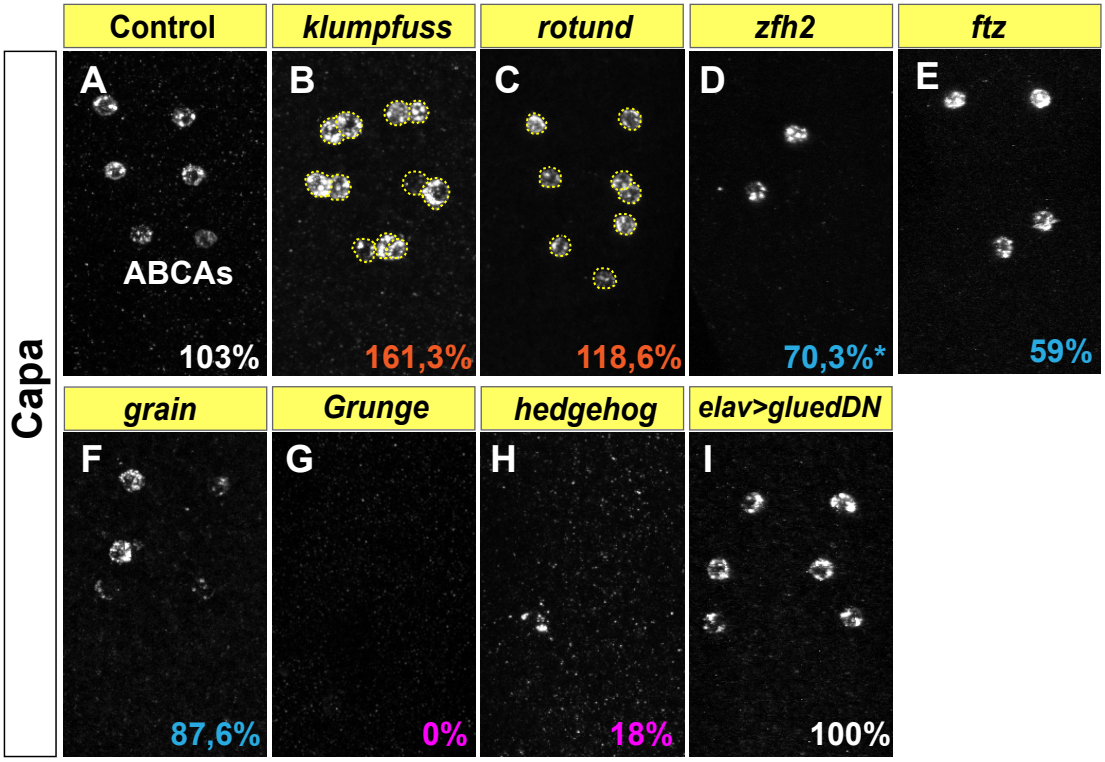
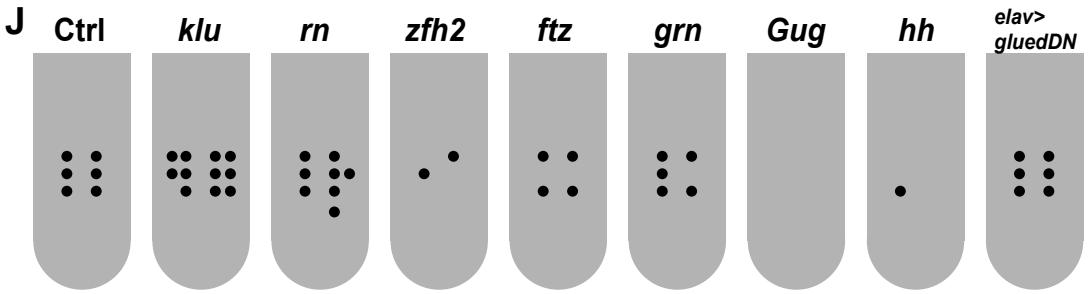

Control
DfH99
elav>p35
cas>p35
A
B
C
D
A5
A6
A7
Capa
cas>numb
cas>Notch intra
numb
E
F
G
H
* p<<0.001
† p<0.05
# ABCAs cells
10.0
7.5
5.0
2.5
0.0
Control
H99
elav>p35
cas>p35
cas>numb
cas>Nintra
numb
I
Ctrl
DfH99
elav> p35
cas> p35
cas> numb
cas> Notch intra
numb

Figure 5


Control
klumpfuss
rotund
zfh2
ftz
Capa
A
ABCAs
103%
B
161,3%
C
118,6%
D
70,3%*
E
59%
grain
Grunge
hedgehog
elav>gluedDN
F
87,6%
G
0%
H
18%
I
100%


J
Ctrl
klu
rn
zfh2
ftz
grn
Gug
hh
elav>
gluedDN

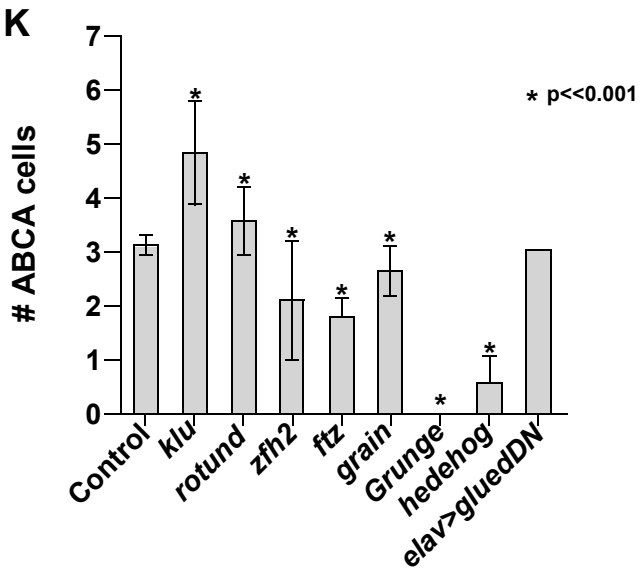
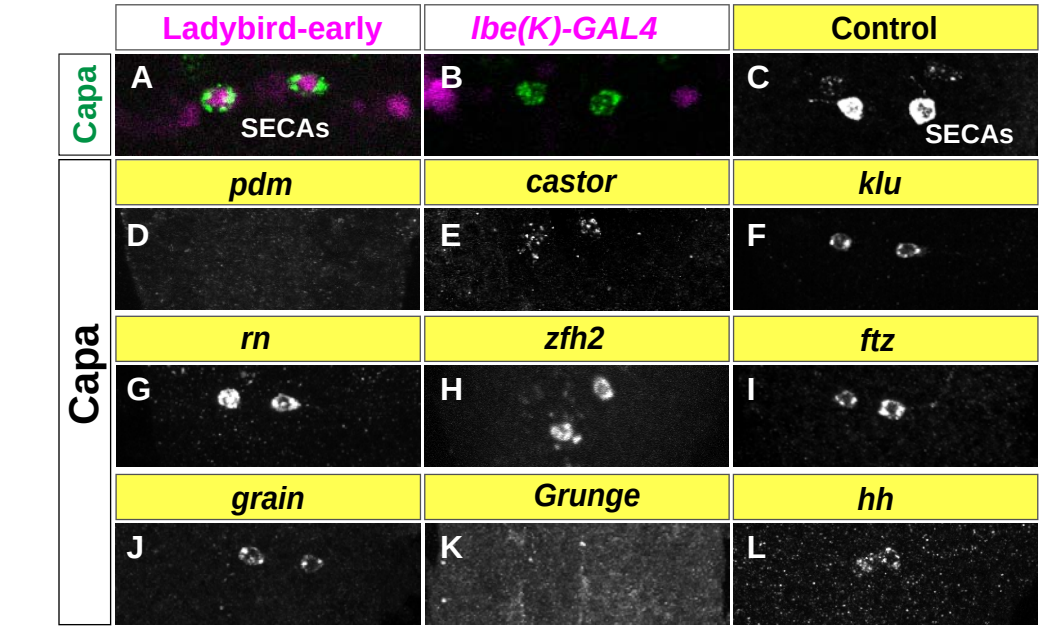
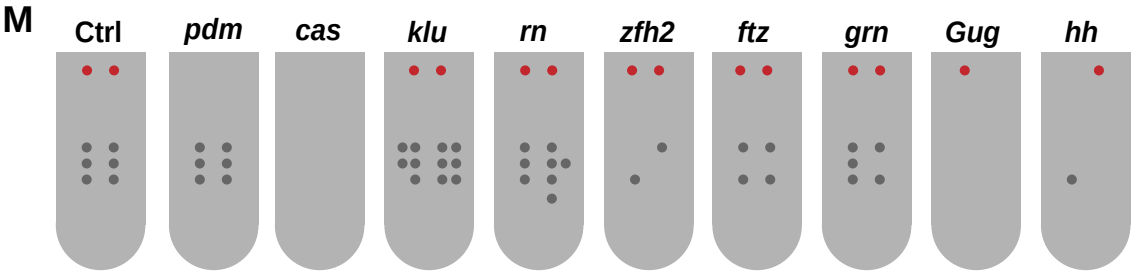
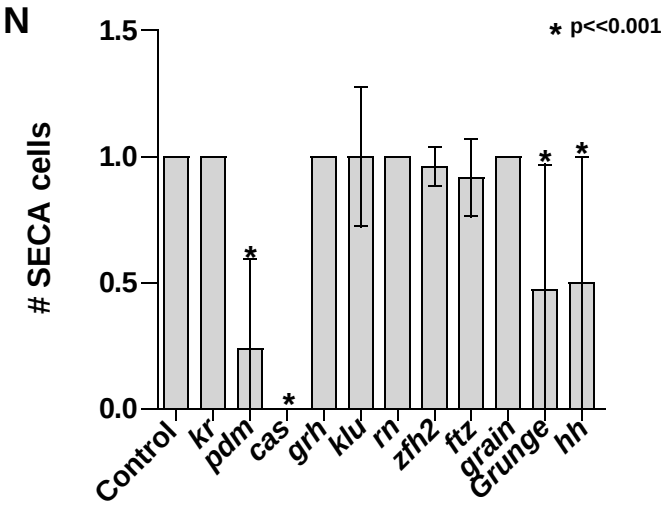

K
ABCA cells
* p<<0.001
Control
klu
rotund
zfh2
ftz
grain
Grunge
hedehog
elav>gluedDN

Figure 6


Ladybird-early
lbe(K)-GAL4
Control
Capa
A
SECAs
B
C
SECAs
Capa
pdm
castor
klu
D
E
F
rn
zfh2
ftz
G
H
I
grain
Grunge
hh
J
K
L


M
Ctrl
pdm
cas
klu
rn
zfh2
ftz
grn
Gug
hh


N
* p<<0.001
# SECA cells
1.5
1.0
0.5
0.0
Control
kr
pdm
cas
grh
klu
rn
zfh2
ftz
grain
Grunge
hh

Supplementary Material (Artwork Files)

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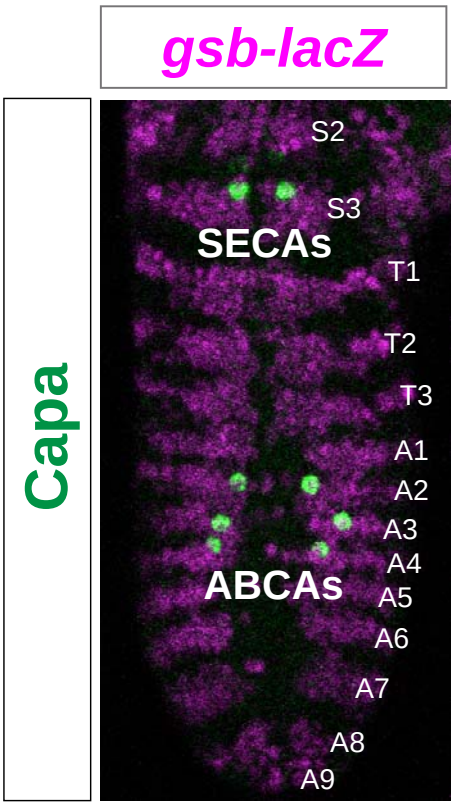
Figure S1



Supplementary Material (Artwork Files)

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Figure S2

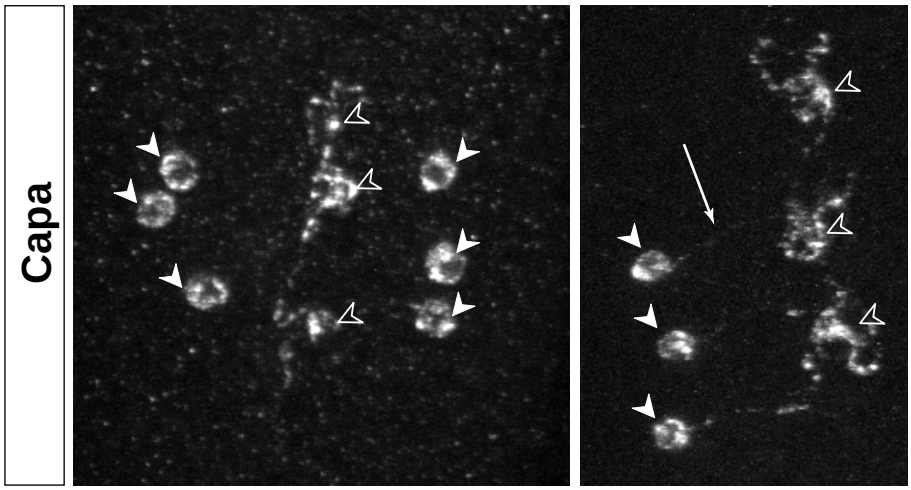

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Figure S3