

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22

Title page

**ASC proneural transcription factors mediate the timely initiation of the neural  
program during neuroectodermal to neuroblast transition  
ensuring progeny fidelity**

Vasiliki Theodorou<sup>1\*</sup>, Aikaterini Stefanaki<sup>1,2</sup>, Minas Drakos<sup>1,2</sup>, Dafne Triantafyllou<sup>1,2</sup>  
and Christos Delidakis<sup>1,2,\*</sup>

<sup>1</sup> Institute of Molecular Biology & Biotechnology, Foundation for Research & Technology  
Hellas, 70013 Heraklion, Crete, Greece

<sup>2</sup> Department of Biology, University of Crete, 70013 Heraklion, Crete, Greece

\*Correspondence: [v.theodorou@imbb.forth.gr](mailto:v.theodorou@imbb.forth.gr), [delidaki@imbb.forth.gr](mailto:delidaki@imbb.forth.gr)

Keywords: proneural factors, achaete-scute complex (ASC), ASCL, chromatin, enhancers,  
neural stem cell, neurogenesis, neuroblast, CHIPseq, histone marks

23 **ABSTRACT**

24 **Background.** ASC/ASCL proneural transcription factors are oncogenic and exhibit impressive  
25 reprogramming and pioneer activities. In both *Drosophila* and mammals, these factors are  
26 central in the early specification of the neural fate, where they act in opposition to Notch  
27 signalling. However, the role of ASC on the chromatin during CNS neural stem cells birth  
28 remains elusive.

29 **Results.** We investigated the chromatin changes accompanying neural commitment using an  
30 integrative genetics and genomics methodology. We found that ASC factors bind equally  
31 strongly to two distinct classes of cis-regulatory elements: open regions remodeled earlier  
32 during maternal to zygotic transition by Zelda and Zelda-independent, less accessible regions.  
33 Both classes cis-elements exhibit enhanced chromatin accessibility during neural specification  
34 and correlate with transcriptional regulation of genes involved in many biological processes  
35 necessary for neuroblast function. We identified an ASC-Notch regulated TF network that most  
36 likely act as the prime regulators of neuroblast function. Using a cohort of ASC target genes,  
37 we report that ASC null neuroblasts are defectively specified, remaining initially stalled,  
38 lacking expression of many proneural targets and unable to divide. When they eventually start  
39 proliferating, they produce compromised progeny. Generation of lacZ reporter lines driven by  
40 proneural-bound elements display enhancer activity within neuroblasts and proneural  
41 dependency. Therefore, the partial neuroblast identity seen in the absence of ASC genes is  
42 driven by other, proneural-independent, cis-elements. Neuroblast impairment and the late  
43 differentiation defects of ASC mutants are corrected by ectodermal induction of individual ASC  
44 genes but not by individual members of the TF network downstream of ASC. However, in wild  
45 type embryos induction of individual members of this network induces CNS hyperplasia,  
46 suggesting that they synergize with the activating function of ASC to establish the chromatin  
47 dynamics that promote neural specification.

48 **Conclusion.** ASC factors bind a large number of enhancers to orchestrate the timely activation  
49 of the neural chromatin program during neuroectodermal to neuroblast transition. This early  
50 chromatin remodeling is crucial for both neuroblast homeostasis as well as future progeny  
51 fidelity.

52

## 53 **BACKGROUND**

54 The *Drosophila* genome exhibits complex and dynamic developmental chromatin and  
55 transcriptional patterns [1-6]. Due to its compact size enhancer elements are tightly spaced and  
56 utilized by many, ubiquitous and tissue specific transcription factors (TF) [5, 7-11]. For any  
57 given cell-type, specific activators turn on the relevant transcriptional program; while in parallel  
58 repressors suppress transcription of genes related to other lineages or temporally inappropriate  
59 states, ensuring proper differentiation and maturation [12, 13].

60 The achaete-scute complex locus (ASC) encodes four paralogous proneural bHLH transcription  
61 factors, Achaete (Ac), Scute (Sc), Lethal of scute [L(1)sc] and Asense (Ase), which regulate  
62 central (CNS) and peripheral (PNS) nervous system development [14, 15]. They exhibit high  
63 evolutionary conservation to mammalian *ASCLs* in both sequence and proneural function [16-  
64 21]. Although prominent in neurogenesis, they also regulate progenitor cell specification and  
65 function in tissues of endodermal and mesodermal origin [22, 23]. In humans, various studies  
66 highlight their oncogenic involvement in malignancies from different germ layers [24].  
67 Examples include small cell lung carcinomas [25], prostate tumors [26], medullary thyroid  
68 cancers [27], gastroenteropancreatic tumors [28], gliomas, grade II and grade III astrocytomas  
69 and a subset of glioblastoma multiforme [29-33]. Also, their strong reprogramming and pioneer  
70 factor abilities [33-37] attest to their transcriptional activating potency.

71 Within the insects, two ancestral ASC-like proneural factors have been characterized, *ASH*  
72 (Achaete and Scute homologue) and Asense (Ase) [38, 39]. In many insect clades *ASH* genes  
73 have duplicated, whereas *ase* has remained as single-copy. *Drosophilids* three *ASH* genes, *ac*,  
74 *sc* and *l(1)sc*, exhibit a considerable degree of functional redundancy [40, 41]. In the early

75 embryonic neuroectoderm (NE), the naïve CNS primordium, global patterning cues initiate the  
76 expression of the three ASH genes in patches of cells [42, 43]. Within these proneural clusters,  
77 cells are at a cell fate crossroad, become a neural stem cell, "neuroblast" (NB), and delaminate  
78 from the neuroepithelium or remain neuroectodermal and eventually take on the epidermal fate  
79 [44, 45]. This cell fate decision is controlled by a finely tuned interplay between ASH  
80 proneurals and Notch signalling, mostly through its E(spl)s effectors [14, 46]. Newly born  
81 neuroblasts start expressing the fourth paralogue, Ase, and other stem cell markers, and divide  
82 asymmetrically to produce ganglion mother cells (GMC), which divide once to produce  
83 differentiated neurons and glia. Unlike PNS primordia, where activity of proneural genes is  
84 required for precursor specification [15], in ASC-deficient embryos most CNS neuroblasts  
85 delaminate, albeit at approximately 25% smaller numbers [47]. These ASC mutant NBs have  
86 restricted progeny and often die after stage 11 through a wave of apoptosis. It remains largely  
87 unknown how ASC proneurals contribute to CNS neuroblast birth and function at the chromatin  
88 level.

89 Here, we have followed up on early seminal genetic work and addressed this biological process  
90 from a genomics point of view and present novel insights regarding the chromatin changes that  
91 accompany CNS neural stem cell birth in terms of global proneural binding, active histone mark  
92 deposition and transcriptional profiles. Combining these datasets revealed a putative TF-  
93 network of proneural target genes, which are likely to comprise the forefront arsenal ensuring  
94 neuroblast functionality. Notably, ASC mutant neuroblasts undergo NE to NB transition poorly,  
95 remaining in a 'stalled state' characterized by lack of timely expression of many proneural  
96 targets and, importantly, without dividing. Eventually, they overcome this arrest but cannot  
97 sufficiently sustain stem cell competence, evident by the depleted glia and neuronal population  
98 resulting in a highly hypoplastic nerve cord. Therefore, ASH proneurals appear to be largely  
99 dispensable for the NB delamination process, but are required for timely initiation of the neural  
100 stem cell program.

101

102

## 103 **RESULTS**

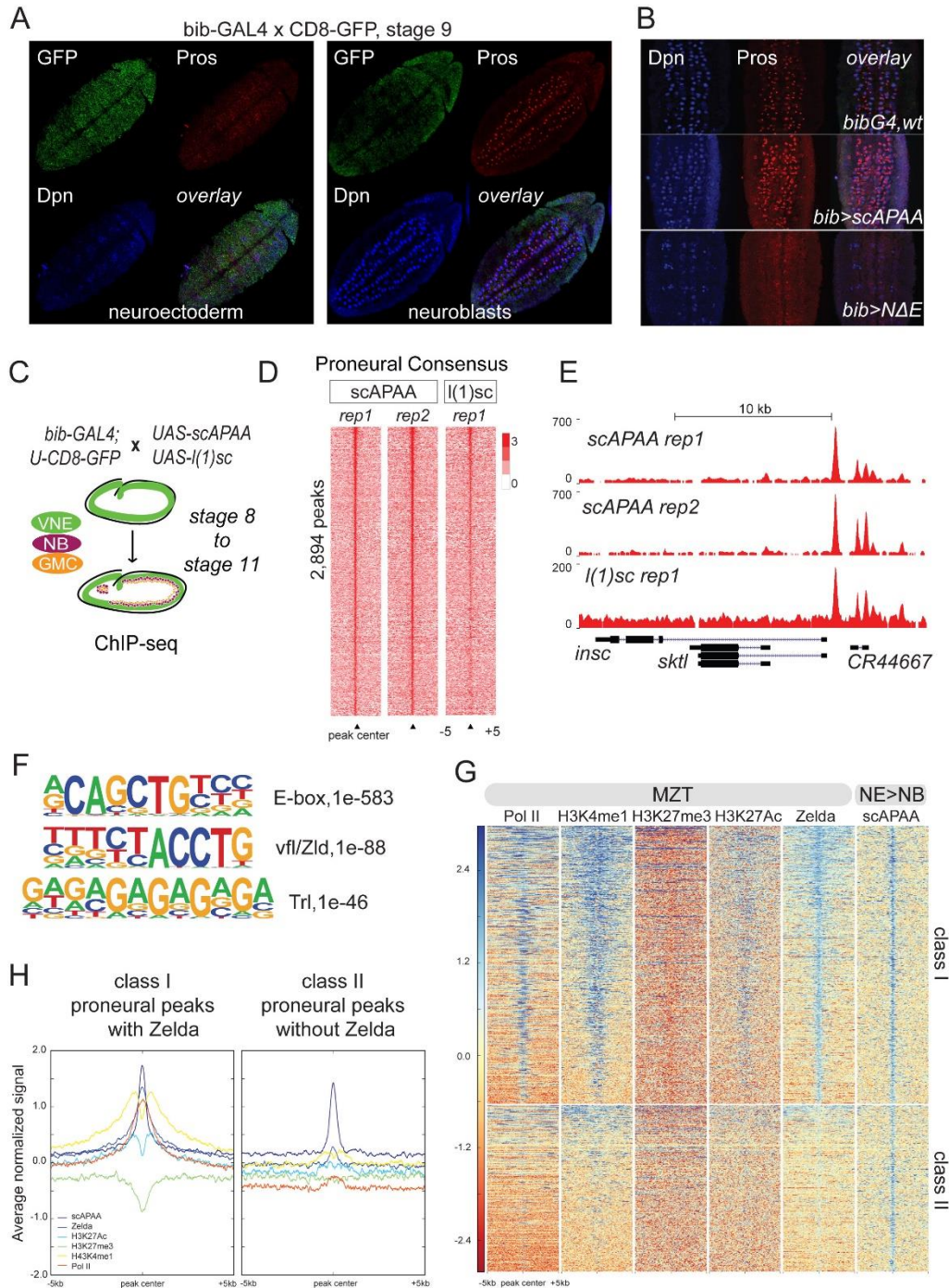
### 104 **Genome-wide mapping of ASH proneural binding during NB specification.**

105 To address the role of the ASH proneural factors, we screened a number of Gal4 lines for  
106 embryonic neuroectodermal expression and selected bib-Gal4 to express myc-tagged variants  
107 of Sc and L(1)sc for genome-wide binding and transcriptome studies. bib-Gal4 is active in the  
108 procephalic and ventral neuroectoderm from stage 8 onwards and by stage 16 GFP is detected  
109 in the ventral nerve cord (VNC) and the mature epidermis (Fig. 1A, Supplemental Fig. S1).  
110 During NB delamination, we detected weak signal in the NBs (Supplemental Fig. S1B),  
111 indicating GFP perdurance rather than active GAL4 expression. bib-Gal4 overexpression of a  
112 wt Sc did not influence NB specification (not shown). However, induction of scAPAA, a  
113 stabilized variant [48], led to a variable, moderate increase in Dpn positive neuroblasts and Pros  
114 positive GMCs progeny (Fig. 1B, middle panel). This subtle increase in the NB/GMC  
115 population led to mild late-stage CNS hyperplasia (Supplemental Fig. S1C) with varying  
116 penetrance and reduced embryonic hatching rate (not shown). On the other hand,  
117 overexpression of an extracellular domain deletion of Notch (UAS-N $\Delta$ ecd, abbreviated U-  
118 N $\Delta$ E), mimicking Notch activation [49] exhibited reduced number of delaminated neuroblasts  
119 (Fig. 1B, bottom panel), severe CNS hypoplasia (Supplemental Fig. S1C-D) and complete  
120 embryonic lethality (not shown). These phenotypes agree with the conventional model of  
121 mutual proneural - Notch antagonism in NB specification, rendering bib-Gal4 an appropriate  
122 driver to monitor the chromatin shifts during NB transition (Fig. 1C).

123 We focused on stage 8- mid 11 encompassing almost the entire duration of neuroblast  
124 segregation and performed three ChIP-sequencing experiments, two against scAPAA and one  
125 against L(1)sc (Fig. 1C). A Venn diagram of called binding events among the three replicates,  
126 as well as the signal intensity heatmaps (Supplemental Fig. S1E), show that ScAPAA and  
127 L(1)sc bind many genomic loci commonly. We derived a consensus of the two ScAPAA  
128 replicates (Supplemental Methods), resulting in 2,894 peaks (Supplemental Table S1). At the

129 level of called peaks, 55% of this strict ScAPAA consensus was also bound by L(1)sc (not  
 130 shown), possibly due to the overall weaker signal in the l(1)sc library (Fig. 1D).

Figure 1



131 An example of common proneural binding is shown for the *insc* locus (Fig. 1E). We will refer  
 132 to this strict, confident consensus of the two ScAPAA replicates as the ‘proneural binding  
 133 consensus’ for the rest of the paper. This proneural consensus showed 27% overlap with Ac

134 modEncode binding [50] and 12% with the Ase-DamID data [51] (Supplemental Fig. S1F).  
135 The limited overlap of ASH proneurals with Ase possibly reflects their expression pattern, since  
136 Ase is expressed solely in the delaminated NBs. De novo motif analysis revealed fine  
137 differences in the E-box motif for each proneural TF (Supplemental Fig. S1F), highlighting  
138 their unique binding preferences beyond their functional redundancy. In addition, we  
139 investigated the binding co-occupancy with Daughterless (Da), a well-described proneural  
140 partner [52] and E(spl)m8, a neuroectodermal specific Notch induced E(spl) repressor that  
141 counteracts proneural/Da function, from modENCODE (Supplemental Fig. S1G). These global  
142 comparisons showed a 15% overlap of proneural consensus with Da and 31 % with E(spl)m8,  
143 while Da exhibited a much higher, 84% overlap with E(spl)m8 binding events. This raises the  
144 possibility that proneurals bind mostly independently of Da and that E(spl)m8 recruitment is  
145 channelled through Da rather than proneural factors.

#### 146 **Proneurals bind developmental DHS regions.**

147 Next, we evaluated the genomic distribution of the proneural binding consensus events and  
148 found high enrichments in upstream regions (Supplemental Fig. S1H), similar to mammalian  
149 Ascl [19] suggestive of an evolutionary conserved positioning of proneural binding motifs close  
150 to gene start sites. De novo motif analysis revealed E-boxes as the primary motif identified in  
151 73% of the proneural peak consensus, followed by the Vfl/Zelda and Trl motifs (Fig. 1F). Zelda  
152 is the pioneer factor that establishes global chromatin organization during the maternal-to-  
153 zygotic transition (MZT) [53-59], which peaks at nuclear cycle 14 (NC14) of stage 5, shortly  
154 before ASH expression in the neuroectoderm. Zelda binding together with profiles of various  
155 histone modification marks and extensive stalled PolIII binding [60-62] has revealed a dynamic  
156 chromatin reorganization in preparation for zygotic transcription. We thus overlapped our  
157 proneural consensus with stage 5 Zld binding events [56] and found a 62% overlap  
158 (Supplemental Fig. S2A), suggesting that at these regions Zelda precedes proneural binding  
159 temporally. We used the two classes of proneural bound regions (class I with Zelda, class II  
160 without Zelda) to investigate the chromatin landscape patterns prior to proneural binding. Based

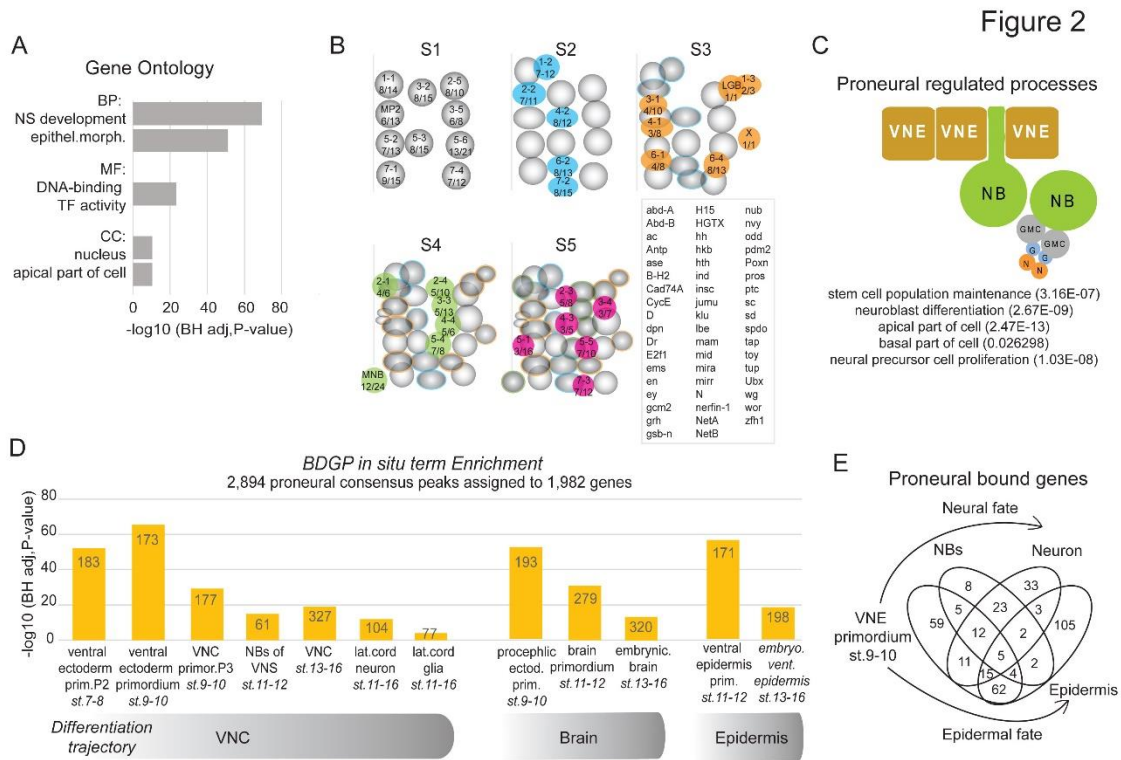
161 on the patterns of H3K4me1 and H3K27Ac, positively associated with chromatin accessibility,  
162 the lack of the repressive H3K27me3, and the PolII signal it appears that prior to proneural  
163 binding class I target regions were nucleosome remodeled and more accessible whereas class  
164 II sites were less accessible. Subsequently, during NB specification proneurals appear to bind  
165 these loci equally strong (Fig. 1G-H). These two classes of cis-elements exhibited differences  
166 in motif enrichment analysis suggesting possible differential TF recruitment (Supplemental  
167 Table S2). Also, class II elements were less frequently located within a 5kb window upstream  
168 from the TSS (Supplemental Fig. S2B) suggesting that they constitute long-range, tissue-  
169 specific enhancers (Reddington 2020).

170 Since regulatory elements correlate with DNase Hypersensitivity Sites (DHS) [8, 11] we  
171 investigated proneural binding occurrence within stage specific DHS and found striking  
172 overlaps (Supplemental Fig. S2C-D). Notably, 89% of proneural binding events were within  
173 DHS from all stages, with higher overlaps in stages 9-11 in agreement with proneural activity  
174 during NB specification. The vast majority, 98%, of class I proneural events were within DHS  
175 (Supplemental Fig. S2C), while class II exhibited a smaller overlap at 74% (Supplemental Fig.  
176 S2C). Importantly, Class I elements were open from st5 onwards, whereas Zelda-independent  
177 Class II elements were more dynamic, becoming more accessible as embryos progress from st5  
178 to st11, perhaps as a result of proneural pioneer activity in preparation for the neural-specific  
179 transcriptional program.

#### 180 **Proneurals target a plethora of genes necessary for proper NB homeostasis.**

181 We then assigned the proneural consensus binding events to 1,983 genes and used the Flymine  
182 tool [63] for downstream mining (Supplemental Table S3). Gene Ontology analysis (Fig. 2A)  
183 showed high enrichments for nervous system development and DNA-binding transcription  
184 factors. 53 members of the Homeobox-like domain superfamily, 69 Zinc finger C2H2-type and  
185 21 Helix-loop-helix DNA-binding domain superfamily genes were amongst the proneural  
186 targets, suggesting proneural regulation of a broad network of transcription factors.





187

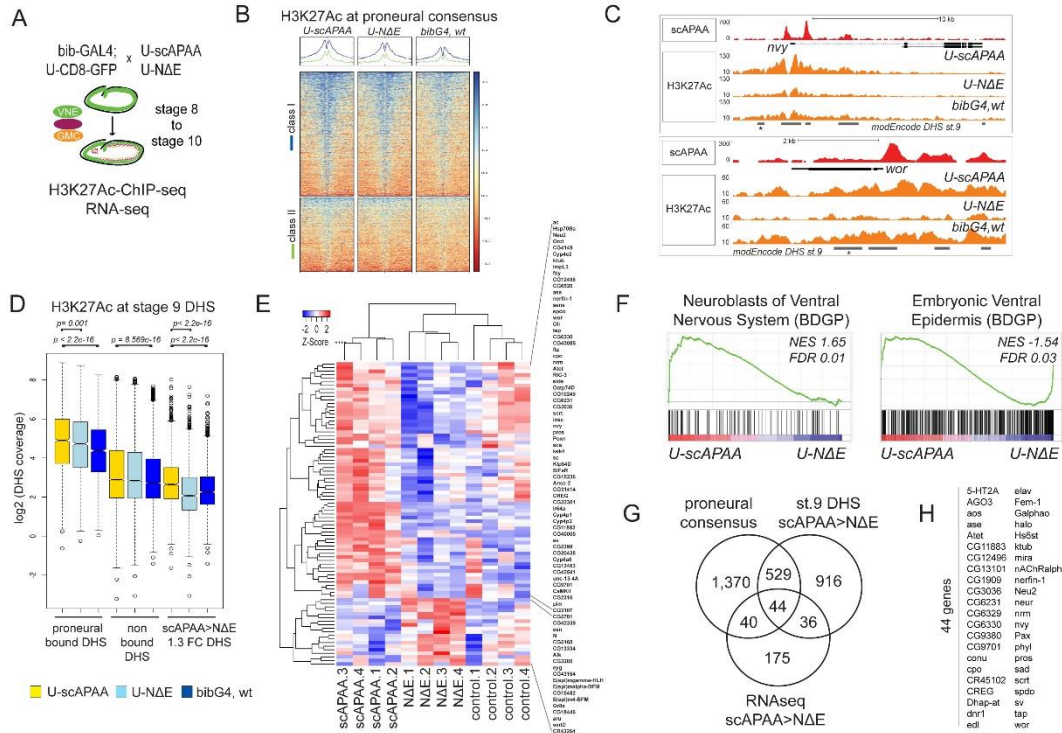
188 Next, we extracted from Flybase [64] genes associated with each specific neuroblast and found  
189 proneural binding in 53 out of 98 neuroblast markers, of all five waves of neuroblast  
190 specification (Fig. 2B). Besides genes that presumably provide neuroblast identity (stemness),  
191 many different processes are needed for proper NB function: delamination; establishment of  
192 cytoplasmic asymmetry, expression and correct segregation of pro-differentiation factors, self-  
193 renewal and proliferation through multiple asymmetric divisions and temporal progression of  
194 progeny types [14, 65]. Notably, proneural target genes fell in all above-mentioned processes.  
195 For instance, the known stem cell identity markers *wor*, *dpr*, *scrt*, *klu*, the temporal genes *hb*,  
196 *Kr*, *nub*, *grh* [66], genes encoding myosin contractile machinery important for delamination,  
197 like *zip*, *sqh*, *Rok* and *Rho1* [67], the cell cycle genes *cycE*, *E2F1* and *stg* and members of  
198 apico-basal polarity organizing Par complex (*baz*), Pins complex (*insc*, *loco*, *mud*, *cno*), the  
199 Centrosome organizing center (*ctp*, *mud*) and the basal compartment (*mira*, *brat*, *pros*) [68].  
200 Thus, proneurals appear to regulate besides delamination many biological processes needed for  
201 neuroblast homeostasis (Fig. 2C).

202 In addition, we investigated the expression patterns of the proneural-targeted genes using the  
203 BDGP *in situ* RNA database integrated in the Flymine tool (Fig. 2D, Supplemental Table S3).  
204 We found that many target genes express in the ventral ectoderm primordium, but also in brain,  
205 VNC, midline and sensory primordia at the time of neural specification. We also found binding  
206 near genes expressed in later developmental stages, in differentiated cell types such as neurons  
207 and glia, also supported by the GO enrichments in neuron differentiation [GO:0030182] and  
208 axonogenesis [GO:0007409] (Supplemental Table S3). A venn diagram of proneural-bound  
209 genes, expressed in the ventral ectoderm, NB, VNC neurons and epidermis (BDGP), showed  
210 common as well as unique genes per cell type (Fig. 2E). Thus, we speculate that besides  
211 orchestrating the neuroblast program, during the NE to NB transition, proneurals may remodel  
212 chromatin in preparation for more committed differentiation states.

### 213 **Proneural binding enhances chromatin acetylation.**

214 Next, we asked whether proneural activity affects chromatin organization in terms of enhancer  
215 remodeling and transcriptional output. For this reason, we generated four replicated RNA-seq  
216 experiments and an H3K27Ac ChIP-seq dataset from staged embryos (Fig. 3A). We restricted  
217 the time window for these experiments by 1 hour (stage 8-mid 10) compared with the proneural  
218 ChIP-seq datasets, to ensure monitoring the initial process of NE→NB specification and dilute  
219 out possible signal from more differentiated cell types. First, we focused on the proneural peak  
220 consensus and found higher H3K27Ac signal in the U-scAPAA embryos, in both class I and  
221 class II regions (Fig. 3B). Importantly, class II elements, which at NC14 exhibited overall low  
222 accessibility, had undergone nucleosome remodelling by st10 (compare the shapes of averaged  
223 signal in NC14 Fig. 1H to Fig. 3B) and exhibited increased H3K27Ac signal in UAS-scAPAA  
224 embryos compared to wt or UAS- $\Delta E$ . Genomic snapshots at *wor* and *nvy*, two bona fide  
225 neuroblast markers [69, 70] are representative examples (Fig. 3C). Along this line, analysis of  
226 H3K27Ac mark on st9 DHS sites, revealed increased signal in the proneural-bound DHS  
227 regions (left panel) compared to the non-bound DHSs (middle panel) (Fig. 3D and  
228 Supplemental Fig. S2E). This indicates that *Drosophila* proneurals elicit nucleosome

Figure 3



229

230 remodeling and enhance active chromatin conformation, consistent with the pioneer function  
 231 of mammalian homologues [36, 37].

232 We subsequently asked which DHSs were most affected in scAPAA vs. NΔE conditions, as a  
 233 way to monitor the neuroblast versus epidermal cell fate selection during lateral inhibition.

234 1,889 loci exhibited more than 30% positive difference in active histone deposition in scAPAA  
 235 versus NΔE overexpressing embryos (Fig. 3D, right panel, and Supplemental Fig. S2E). These

236 genomic sites were near 1,525 genes, enriched in ventral ectoderm and nervous system related  
 237 genes (not shown), similar to the proneural consensus distributions of Fig. 2D. However, only

238 16%, (306 sites), of the affected DHSs coincided with proneural binding (not shown). The  
 239 remaining not-bound DHSs were close to proneural-bound genes, 39% overlap at the gene

240 assignment level, which indicates that proneural binding has broader effects outside its binding  
 241 element, either as a result of gene transcription or long-range looping interactions (note the non

242 bound DHSs with \* at the *wor* and *nvj* examples in Fig. 3C). Alternatively, these differentially  
 243 acetylated DHSs may represent cis-elements regulated by Notch signalling independently of

244 ASH activity.

245 **Combination of transcriptome and chromatin profiling reveals putative core regulators**  
246 **of neural stem cell function.**

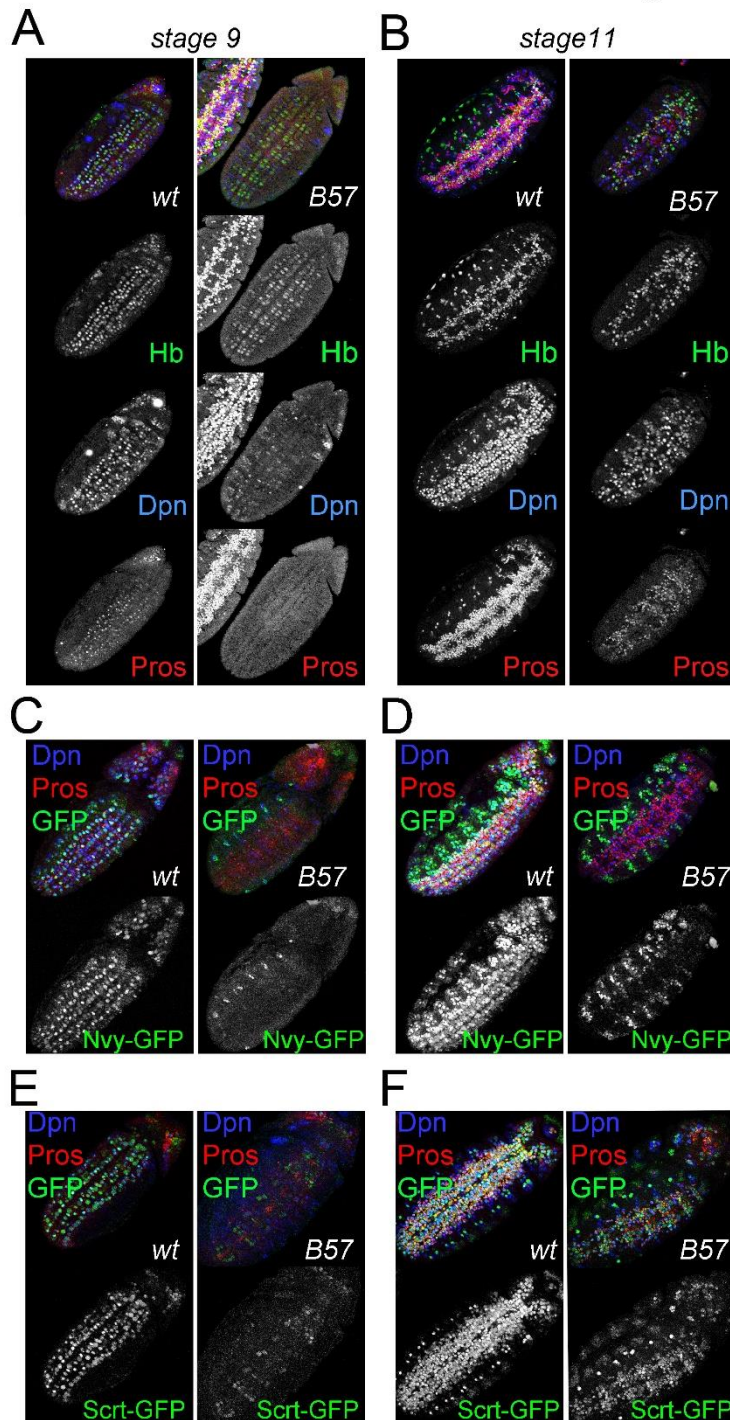
247 To identify the transcriptional changes that accompany neural selection, we performed RNAseq  
248 expression profiling (Supplemental Table S5). In the differentially expressed genes (DEG)  
249 between the U-scAPAA and U-NΔE embryos (FDR<0.2, p<0.0025) (Fig. 3E) there were many  
250 neurogenesis related transcription factors. Indeed, the ranked genes clearly mirrored the neural  
251 versus epidermal fate specification that proneurals and Notch favor respectively (Fig. 3F). In  
252 addition, we found significant enrichments with the class II proneural binding events  
253 (Supplemental Fig. S2F) as well as with the affected DHSs in H3K27Ac deposition in  
254 scAPAA>NΔE (Supplemental Fig. S2G). These correlations demonstrate that the regulatory  
255 elements filtered out from the above integrative genomics analyses are transcriptionally  
256 relevant. To expand on this observation, we overlapped (a) genes with higher RNA expression  
257 in U-scAPAA versus U-NΔE embryos (with the significance threshold relaxed to p<0.05), (b)  
258 genes that exhibited proneural binding and (c) genes with differential H3K27Ac in nearby  
259 DHSs (Fig. 3G). We found that 40% of DEGs were associated with the one or/and the other  
260 dataset. In Fig. 3H we present the 44 genes from the intersection of the three. In this high-  
261 confidence gene set we find *ase*, *nerfin-1*, *sv*, *tap*, *pros*, *sens*, *scrt*, *wor* and *navy*, known to act  
262 in the CNS, PNS and midline. Thus, this TF network regulated by proneural and Notch interplay  
263 could be the initial battery of factors required to sustain neural precursor functionality.

264

265 **ASC mutant neuroblasts are temporarily stalled and devoid of stem cell identity markers.**

266 ASC null (Df(1)scB57) embryos show a reduced number of delaminated NBs and a drastic  
267 reduction of mature neurons [47]. However, it has not been documented in detail how these  
268 mutant NBs behave. For this purpose, we selected 13 TFs (Dpn, Klu, Wor, Sna, Esg, Scrt, Nvy,  
269 Pros, Hb, Kr, Nerfin-1, Tap, Oli), whose genes exhibited proneural regulation in our genomic  
270 analyses, and examined their expression in wt vs ASC null embryos. In wt embryos all 13  
271 display NB expression to some extent. A summary of their genomic features and expression  
272 patterns is in Supplemental Table S6.

Figure 4



273

274 Unexpectedly, in the ASC deletion we observed that delaminated neuroblasts are temporarily  
275 stalled during stages 9 and 10. They do not express the stem cell specific markers Dpn (Fig.  
276 4A), Wor (Figure S3A), Nvy (Fig. 4C), Scr (Fig. 4E), Klu and Oli (not shown), compared to  
277 wt NBs which during this time robustly express all these TFs. In contrast, the expression of Hb

278 (Fig. 4A), Sna, Esg and Kr (not shown), appeared unaffected in the mutant NBs. Significantly,  
279 mutant neuroblasts did not proliferate, evident by the lack of Pros positive GMCs (Fig. 4A) and  
280 phosphoH3-S10 (pH3) mitotic events (Supplemental Fig. S3B). We used the UAS-FUCCI, a  
281 GFP-E2F1 and RFP-CycB dual expressing system, that allows cell cycle monitoring by fusing  
282 cell-cycle specific degrons to fluorescent proteins [71]. Consistent with bib-Gal4 activity  
283 specifically in the NE, wt NBs showed little or no accumulation of FUCCI signal (Supplemental  
284 Fig. S3C). Stalled ASC NBs, however, accumulated both these markers demonstrating a G2/M  
285 arrest, suggesting that after delamination they retained the NE-expressed FUCCI signal since  
286 they had not divided yet (Supplemental Fig. S3D). These results suggest that ASC deficient  
287 neuroblasts undergo NE to NB transition poorly as they do not proliferate, nor initiate  
288 expression of the entire neural TF program (Supplemental Fig. S3E).

289 Despite this early developmental arrest, starting at late stage 10/early 11, we observed a gradual  
290 rebound in NB marker expression, accompanied by initiation of NB mitoses. By late stage 11,  
291 mutant NBs started expressing Dpn (Fig. 4B), Scrt (Fig. 4F), Oli (Supplemental Fig. S4), Wor  
292 and Klu (not shown). The only marker that never rebounded, demonstrating obligate ASC NB  
293 regulation, was Nvy (Fig. 4D). Hb (Fig. 4B) and Sna (not shown), not affected at earlier stages,  
294 were turned off as usual at this late stage, while Kr continued expressing from earlier stages as  
295 normal (not shown). Concomitantly, many GMCs were born, albeit with an aberrant molecular  
296 profile. These GMCs expressed Pros (Fig. 4B), Scrt (Fig. 4F), Esg, Hb (subset, Fig. 4B), Kr  
297 (subset, not shown), Oli (subset, Supplemental Fig. S4A) and Nerfin-1 (subset, Supplemental  
298 Fig. S4B), but not Nvy (Fig. 4D) or Tap, which is mostly expressed in a large subset of GMCs  
299 (Supplemental Fig. S4C). Tap eventually turned on in GMCs by stage 13 (not shown).  
300 Therefore, the timely expression of neuroblast and GMC markers and proliferation capacity of  
301 neural stem cells is ASC dependent.

### 302 **ASC mutant neuroblasts are defective and produce impaired progeny.**

303 Despite this rebound in mutant NB identity, late embryos are severely hypoplastic. Staining  
304 with axonal markers revealed a fragmented nerve cord, a complete lack of the three VNC  
305 longitudinal nerve tracts and severe defects in intersegmental/segmental nerves (Supplemental

306 Fig. 5A), see also [47, 72]. Axonogenesis is normally guided by communication cues between  
307 neurons and glia from the CNS, PNS and midline [73-78]. Glia play a crucial role both in  
308 prefiguring axonal paths and in providing trophic support to neurons. This is evident in glia  
309 depleted, *gcm* mutant embryos [79], where longitudinal nerve tracts also fail to develop. We  
310 found a diminished glia population in late ASC embryos. This was more evident in the  
311 abdominal segments, by an at least 70% reduction in Repo positive glia (Supplemental Fig.  
312 5B). Specifically, the two characteristic continuous columns of longitudinal glia lining the  
313 dorsal side of the developing nerve cord from st13 onwards were depleted. Their longitudinal  
314 glioblast progenitor (LGB), however, was present in many hemisegments earlier (st.10/11) (not  
315 shown); suggesting that in ASC mutants the born LGB is defectively programmed.

316 Besides gliogenesis, we next assessed the ability of mutant NBs to generate pioneer neurons  
317 that, together with the depleted glia, would explain the lack of longitudinal nerves. It is already  
318 known that two pioneer sibling neurons, *dMP2* and *vMP2* (progeny of *MP2*, an S1 wave NB),  
319 are absent or mis-specified in ASC mutants [80, 81]. We used *Eve* staining, to identify the *aCC*/  
320 *pCC* sibling pioneer neurons (progeny of S1 NB1-1), as well as the U-neurons (S1: NB7-1),  
321 the EL-neurons (S4: NB3-3) and the RP2 motor neuron (S2: NB4-2) (Supplemental Fig. 5C).  
322 ASC stage 11 embryos have only just started producing GMCs, accordingly no *Eve* positive  
323 neurons were seen (not shown), a time when normally the *aCC*/*pCC* pair is formed and  
324 expresses *Eve* and *Fas2* [82]. In later stages, we still failed to detect the *Eve*<sup>+</sup> *aCC*/*pCC* pioneer  
325 neuronal pair (Supplemental Fig. 5C). Only one medial *Eve* positive, *Fas2* negative neuron was  
326 observed (not shown), presumably the RP2. In addition, we observed reduced numbers of EL  
327 neurons and extremely rare U neurons (Supplemental Fig. 5C).

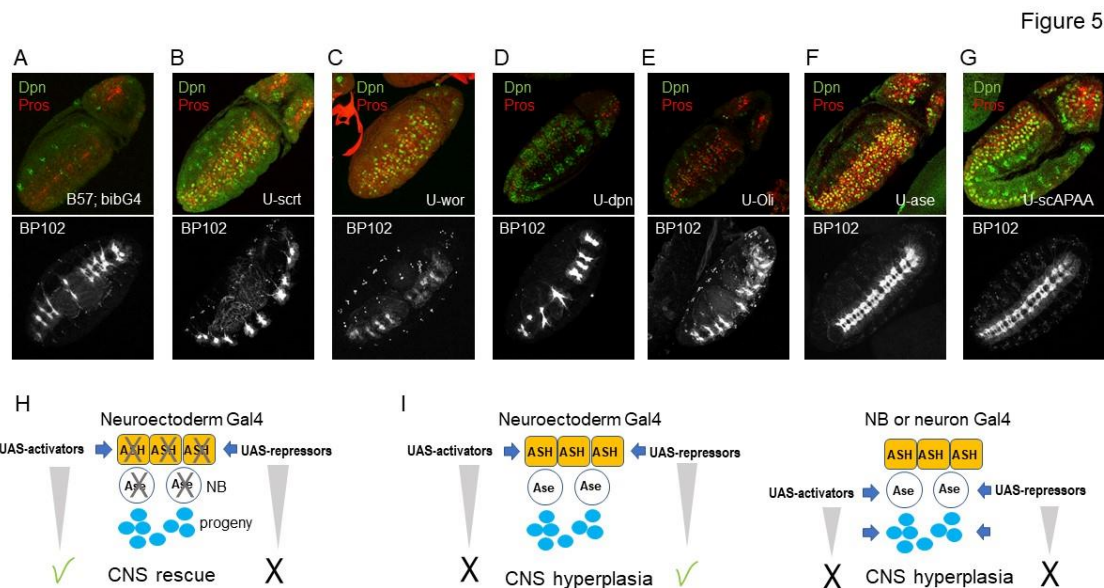
328 Since the four pioneer neurons (*aCC*/*pCC* and *dMP2*/*vMP2*) and the longitudinal glia are born  
329 from precursors specified at the early S1-S3 waves of neurogenesis, we wondered whether ASC  
330 mutants also exhibit defects in neurons/glia born from later NB waves during late st10-11, a  
331 time when mutant NB activity has rebounded. *eagle-lacZ* is a marker of four NBs and their  
332 progeny, three of which arise during S4-S5 (S3:NB6-4, S4:NB2-4, and NB3-3 and S5:7-3) [83].  
333 We observed that in mutant embryos these NBs delaminate and are present in most neuromeres

334 (Supplemental Fig. S6A-B), however, their progeny is variably depleted (Supplemental Fig.  
 335 S6C-D) and their axonal projections deformed, accompanying an anterior to posterior  
 336 commissure (AC-PC) collapse (Supplemental Fig. S6E). Collectively, these observations  
 337 suggest that ASC deficient NBs, both from early and late phases of specification, have an  
 338 inherently defective program and cannot sustain correct progeny differentiation.

339

340 **Ase can substitute for the ASH genes to initiate the neural program in the neuroectoderm**

341 We next investigated whether any of the downstream proneural targets revealed by our genomic  
 342 experiments would be able to rescue the neurogenesis defects of the ASC deficiency, if  
 343 transgenically provided using the neuroectodermal driver bib-Gal4 (Fig. 5). We tested UAS-



344 scr, UAS-wor, UAS-dpn and UAS-Oli, four of the proneural targets that showed a delayed  
 345 onset of expression in the absence of the ASC. None of these was able to rescue NB stalling at  
 346 st9. We observed a detectably earlier rebound of NB activity at early st10, evident by the earlier  
 347 Dpn expression and the emergence of Pros+ GMCs (Fig. 5B-E, top panels). Nonetheless, this  
 348 slight NB rescue was not able to improve the severe late hypoplastic phenotype (Fig. 5B-E,  
 349 bottom row), suggesting that these factors are not capable of activating the full neurogenic  
 350 program in the absence of ASC genes. In contrast, induction of UAS-scAPAA or UAS-ase led  
 351 to a vast improvement in the delamination defect and the timely activation of NBs (Fig. 5F-G,



352 top), which now started dividing normally at st9. At later stages, the VNC was almost complete  
353 with only minor constrictions (Fig. 5F-G, bottom).

354 Therefore, re-instating proneural expression in the neuroectoderm can greatly rescue  
355 neurogenesis demonstrating that the ASH and Ase proteins have equivalent activities, despite  
356 their distinct expression patterns. To clarify this further we used the Df(1)sc19 ASC deficiency  
357 (Supplemental Fig. S7), which deletes *ac*, *sc* and *l(1)sc*, but spares *ase*. In this background, NB  
358 stalling was still evident during stage 9 (Supplemental Fig. S7A). Ase itself also exhibited a  
359 small delay in expression, however its expression preceded Dpn (Supplemental Fig. S7B) and  
360 Pros (not shown), both rebounding a little after Ase expression by early stage 10 (Supplemental  
361 Fig. S7C), earlier than in Df(1)scB57. The late CNS hypoplasia was also improved in  
362 Df(1)sc19. The population of glia was richer (Supplemental Fig. S7D) and the aCC/pCC  
363 pioneer neuron pair was sometimes present (not shown). The VNC had fewer neuromere gaps,  
364 as reported by [72], although the wt pattern of three Fas2-bearing longitudinals was never fully  
365 restored (Supplemental Fig. S7E). Therefore, the endogenous expression of Ase in the  
366 delaminated neuroblasts can greatly improve NB functionality (sc19 vs.B57), but not as  
367 efficiently as when we induce it in the neuroectoderm during NB specification (Fig. 5F),  
368 suggesting that the neuroblast program at the chromatin level commences during the NE to NB  
369 transition.

370 The foregoing experiments demonstrated that although individual ASC proneurals are  
371 sufficient to rescue the CNS defects caused by ASC deletion, none of their other primary targets  
372 tested were competent to do so (Fig. 5B-E). However, in the presence of proneural proteins (in  
373 wt background), *scrt*, *wor* and *dpn* neuroectodermal overexpression by *bib-Gal4* led to  
374 significant neural hyperplasia evident at the level of longitudinal connectives and segmental/  
375 intersegmental nerve bundles (Supplemental Fig. S8A). Cuticle preps showed epidermal holes  
376 (Supplemental Fig. S8B), suggesting that *scrt*, *wor* or *dpn* NE overexpression tipped the balance  
377 in favour of NBs at the expense of epidermis. Although, in the wild type context *bib>scAPAA*  
378 overexpression on its own had a weak effect (Supplemental Fig. S1B-C), coexpression with  
379 *dpn* enhanced the hyperplasia produced by either alone (Supplemental Fig. S8A). Similar

380 enhancement was observed upon co-expressing two proneurals together, scAPAA with l(1)sc  
381 (Supplemental Fig. S8A). Notably, VNC hyperplasia was not seen when these genes were  
382 induced in the neuroblasts by pros-Gal4 (starts expressing in st11 NBs, GMCs and neurons)  
383 (Supplemental Fig. S8C) or in neurons using elav-Gal4 (starts expressing in st13 NBs, GMCs  
384 and neurons, not shown). These results suggest that TFs of the Snail (Wor, Scrt) and Hes  
385 families (Dpn), most known to act as repressors [84, 85], can enhance the NB-promoting  
386 activity of proneural TFs, but have little genuine activating potency to initiate the neural  
387 program on their own (Fig. 5H-I, model cartoons). This conclusion is supported by the ectopic  
388 neural cells in the wing disk induced by a TF cocktail consisting of a proneural (Ase), a Snail  
389 (Wor), as well as two more broadly NE-expressed TFs (SoxN and Kr) [86].

390

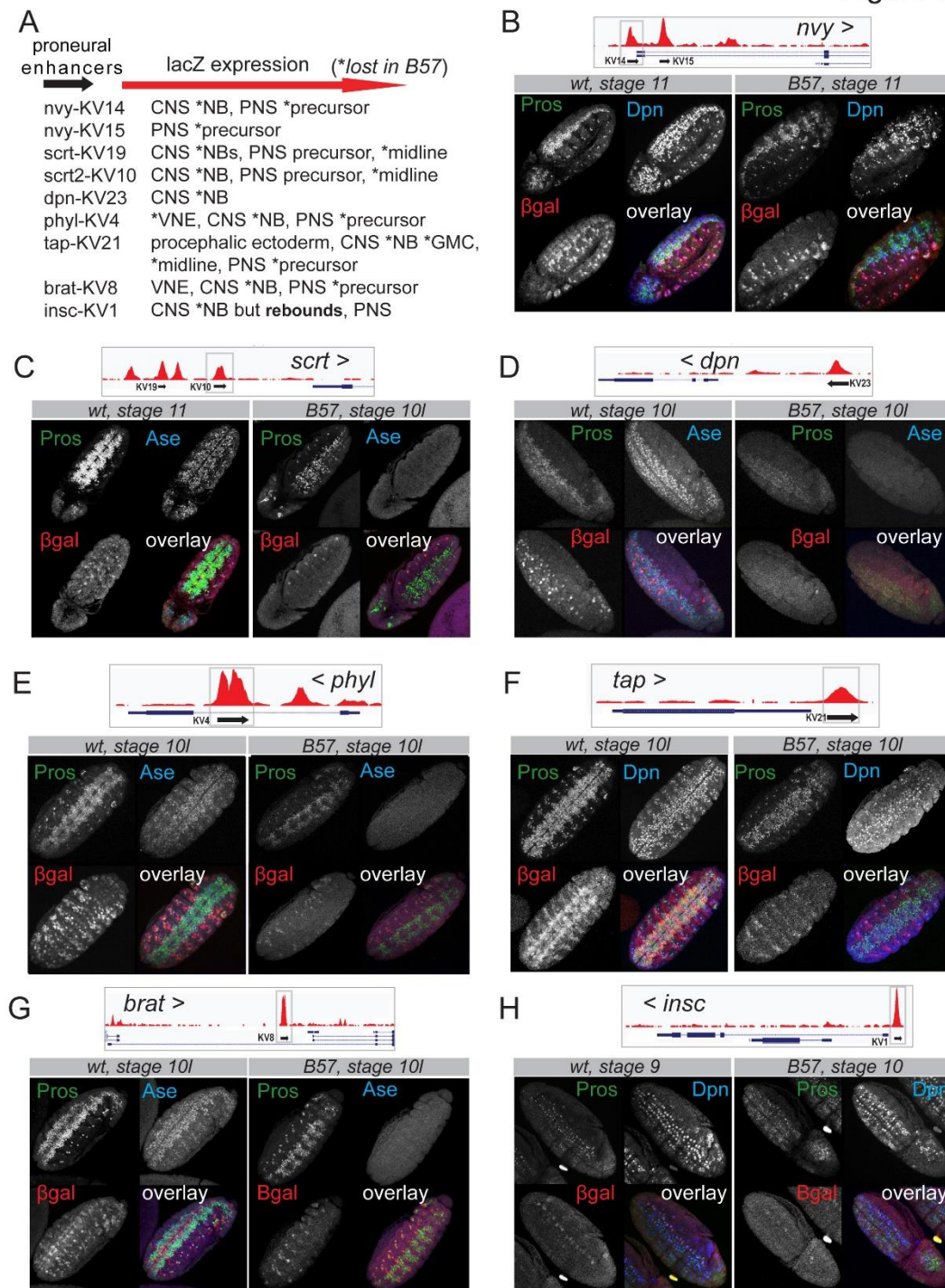
### 391 **Proneural bound cis-elements exhibit enhancer activity and proneural dependency.**

392 To investigate the transcriptional activity of the proneural bound elements we generated 10  
393 transgenic lacZ reporter flies. We selected proneural peaks, near *navy*, *dpn*, *scrt*, *wor* and *tap*  
394 genes, whose protein products showed proneural dependency in mutant embryos in our  
395 foregoing analysis. We included binding events near *insc* and *brat*, two key neuroblast genes  
396 that are implicated in apico-basal polarity and asymmetric cell division [87] and one intronic  
397 peak from the *phyl* gene, a known PNS proneural target [88]. Most of these regions coincided  
398 with DHS sites and half had Zelda binding during MZT (Supplemental Table S7). All fragments  
399 showed enhancer activity in some regions of the developing nervous system, central and/or  
400 peripheral and none in non-neural tissues. The wor-KV29 exhibited weak expression and was  
401 not studied further. For the remaining lines, we compared the lacZ expression patterns in  
402 wildtype and Df(1)scB57 embryos, summarized in Fig. 6A.

403 Briefly, the *navy* enhancers, exhibited different patterns, navy-KV14 had CNS and PNS  
404 expression (Fig. 6B) while navy-KV15 was PNS exclusive (not shown). In mutant neuroblasts,  
405 navy-KV14 expression was abolished throughout neurogenesis similar to the Nvy protein (Fig.  
406 4C-D). In scratch-KV10-lacZ wt embryos, we detected moderate NB and stronger midline  
407 signal, which was lost in mutants (Fig. 6C) in contrast to the rebound in scrt-GFP protein (Fig.

408 4F). *dpn-KV23*, was expressed in S3 and S4 NB waves and by stage 13 had expanded to cover  
 409 the whole NB pool (not shown). In the *Df(1)sc-B57* mutant *KV23* was never activated (Fig.  
 410 6D), in contrast to the resumed *Dpn* protein expression (Fig. 4). The *phyl-KV4* enhancer  
 411 expressed from *st9/10* in NBs and some VNE clusters (Fig. 6E). Next, *tap-lacZ*, exhibited  
 412 ectodermal, CNS (subset of NBs and GMCs) and PNS expression (Fig. 6F). In early mutant  
 413 embryos, the NB/GMC expression was lost (Fig. 6F) but we did detect limited expression in

Figure 6



414 GMCs and midline from stage 13-14 onwards (not shown). Similarly, *brat-lacZ* (Fig. 6G)  
415 exhibited broad neuroblast expression in wt embryos but its expression was lost in the mutant  
416 background, even after the onset of asymmetric divisions and generation of Pros positive GCM  
417 progeny. Lastly, the *insc-KV1* enhancer showed extensive NB expression from S1-S2 onwards  
418 with an emphasis in the lateral and intermediate rows. It exhibited absence of expression in  
419 mutant NBs during the stalling window but did express during the rebounding period (Fig. 6H).  
420 Thus, with the sole exception of the *insc* enhancer, the NB-specific activity of *navy*, *scrt*, *dpn*,  
421 *phyl*, *brat* and *tap* regulatory elements exhibited absolute ASC dependency both during stalling  
422 as well as after stem cell activity resumption. This suggests that, at the chromatin level, the  
423 delayed NB activation in the absence of proneurals is mediated by cis-elements distinct from  
424 those bound by proneural proteins. Unlike NB expression, all enhancers that drove PNS  
425 expression displayed activity in the *Df(1)scB57* mutant in the ASC-independent sensory organs  
426 [89], most likely due to the activity of the *atonal* and *amos*, proneural factors exclusive to PNS  
427 primordia [90, 91].

428

## 429 **DISCUSSION**

430 **Chromatin dynamics during embryonic nervous system development.** The advent of  
431 genomics has revealed that the chromatin of any given cell is a blueprint of past, current and  
432 future maturation states both in homeostasis and disease [92-96]. In *Drosophila* as well, studies  
433 of cell fate transitions depict dynamic chromatin shifts during development [2-5, 8, 97-99].

434 By mapping ASH binding events during neural stem cell specification, we found a high co-  
435 occurrence with accessible regions pre-modelled during MZT, a time when Zelda is crucial for  
436 establishing chromatin organization for subsequent tissue-specific transcription [57, 100].  
437 Since ASH proneurals are amongst the earliest zygotically transcribed genes [54, 101], we  
438 hypothesize that they may survey the early gastrula chromatin to gain access to neurogenesis  
439 related enhancers and possibly pre-initiate target transcription. This notion is supported by a  
440 single-cell RNAseq study of the early gastrula where the neuroectoderm primordium cell

441 cluster expressed sc and some of its direct targets as identified here [102]. Later in the mature  
442 neuroectoderm, we demonstrate that proneurals also bind Zelda-independent elements, which  
443 showed restricted accessibility at the onset of zygotic transcription. ASH binding at these  
444 enhancers and concomitant gain in histone activation marks near known neural stem cell genes  
445 demonstrates their activating potency.

446

447 **ASC proneurals mediate the timely activation of the neural stem cell program in the**

448 **neuroectoderm:** Our work indicates that during NE to NB specification spanning stages 8-11,  
449 proneural-mediated chromatin reorganization and transcription is essential for the proper later  
450 unfolding of the entire NB lineage. For the first time we demonstrate that proneurals establish  
451 NB homeostasis of all 5 delamination waves, based on our genomic data (Fig. 2B), the  
452 phenotypic analysis of mutant NBs, both early (Fig. 4) and late born (Supplemental Fig. S6)  
453 and the expression patterns of the cloned proneural enhancers in vivo (Fig. 6). Thus, as reported  
454 for a single neuroblast, the MP2 [80, 81], it appears that all NBs that manage to delaminate in  
455 ASC mutants are mis-specified and cannot overcome functionally the initial stalling.  
456 Interestingly, murine *Ascl1* depleted neural precursors also exhibit a similar delay [103].

457 Although proneural factors are crucial in the timely execution of the NB transcriptional  
458 program, partial activation of the program happens in their absence (Fig. 4). This is most likely  
459 mediated by different enhancers than those bound by ASH proteins (Fig. 6). The elusive  
460 proneural factors in ASC null embryos have been a long-standing puzzle [47, 104]. Such TFs  
461 could be Hb, in collaboration with Sna [105], since the expression of both was unaffected by  
462 ASC loss (Fig. 4A). Another possibility would be Daughterless, which heterodimerizes with  
463 ASH proteins, but also functions as a homodimer [52, 106]. Earlier observations have shown  
464 that L(1)sc and Ase can bind DNA as homodimers in vitro [107]. From the narrow overlap of  
465 our proneural binding consensus with Da (Supplemental Fig. S1G) it seems that in the  
466 embryonic neuroectoderm the two act to a large extent via distinct enhancers, contrary to the  
467 current belief that proneural factors are obligate heterodimeric partners of Da. This also agrees

468 with the strong enhancement of the neural hypoplasia of double *ASH* and *Da* mutants [47]. On  
469 the other hand, it is unlikely that *Wor* and *SoxN* are the compensating proneural TFs as  
470 proposed by [104]. That study demonstrated that *Wor* and *SoxN* use their repressive capacities  
471 to promote neurogenesis, since *EnR* (Engrailed repression domain) fusions phenocopied their  
472 effect upon ectopic expression in epithelial cells [86]. It is unlikely that a duo of repressors  
473 would be able to activate the large cohort of NB specific genes that seems to be turned on by  
474 proneural factors (our study). In fact we have shown that *wor* is under *ASH* transcriptional  
475 control (Fig. 3C, Supplemental Fig. S3A) and reinstating its expression in *ASH* mutants is  
476 insufficient to rescue the CNS hypoplasia (Fig. 5C), although it mildly improves NB recovery.  
477 Regardless of the identity of other NB-promoting TFs, the eventual initiation of proliferation  
478 and rebound in the expression of key identity genes in *ASH* deficient NBs is insufficient to  
479 restore neural programming at the organism level, as evidenced by the depleted neuronal/glia  
480 progeny. This suggests that the *ASH* TFs are vital for neural stem cell homeostasis.

481

482 **Networks downstream of proneurals.** Integration of the proneural binding events with the  
483 RNAseq and H3K27Ac changes during Notch mediated lateral inhibition revealed a  
484 downstream TF network, likely to consolidate the neural cell fate. *Ase* plays a central part in  
485 this network as being the only NB-specific TF with potent activating function [108]. The  
486 overlap of NE-expressed *ASH* binding events with NB-expressed *Ase* binding suggests that in  
487 the neuroectoderm *ASH* proneurals may mark neural enhancers which *Ase* will subsequently  
488 sustain to unfold the NB program. This is demonstrated in the *sc19* deficiency where the  
489 presence of *Ase* partially improves mutant NB functionality and progeny development,  
490 compared to the deletion of all four *ASH* members (Supplemental Fig. S7). However, we find  
491 it impressive that the neuroectodermal ectopic induction of *Ase* can almost fully rescue the  
492 neurogenesis defects (Fig. 5F), proving, first, its functional equivalence to *ASH* TFs and,  
493 second, that the neural program must be installed early on during neural stem cell selection.  
494 The remaining TFs of this network are in their vast majority transcriptional repressors,  
495 highlighting the importance of blocking alternative transcriptional programs and differentiation

496 fates to ensure proper unfolding of the NB program. We show that single members of this  
497 network contribute to neurogenesis, but we believe they mainly work combinatorically and in  
498 parallel to an ASC factor [86]. Snail TFs are central in this network and appear to have pivotal  
499 roles in NS development [70, 105, 109]. Snails however are not essential for NB ingression  
500 [67], instead, it seems that they regulate NB function and GMC transition [105, 110]. In addition  
501 to these core downstream TFs, NE proneurals bind near >1000 genes, which may contain  
502 previously uncharacterized players in implementing the NB fate and launching the subsequent  
503 GMC and neuron/glia developmental programs.

504

505 **Proneurals pioneer differentiation programs partly in the stem/progenitor cell:** The  
506 mature VNC is the outcome of a complex crosstalk of glia and neuron signaling originating in  
507 the CNS, midline [75] and PNS [76]. Our identified proneural binding events near genes of all  
508 nervous sub-systems validate the genetic evidence of ASC involvement in their development  
509 [42, 47, 89, 111]. We thus propose that the late CNS defect in ASC embryos is the collective  
510 outcome of impaired stem cell specification and impaired progeny from different sub-systems,  
511 failing to establish the necessary communication cues.

512 In addition, studies in flies and mice have shown that, besides stemness, proneurals impact  
513 neuronal differentiation as well [112-116]. In our work, we identified binding near genes  
514 expressed in later differentiated cell types, GMC, neurons and glia (Fig. 2), where ASC gene  
515 expression has been extinguished. For at least one of these genes, *tap*, we showed that its protein  
516 expression is greatly compromised in ASC mutant GMCs (Supplemental Fig. S4C). We  
517 envision that this is happening in two ways: First, proneurals could regulate chromatin  
518 dynamics at neuronal/glia enhancers during neuroblast specification but robust transcriptional  
519 activation only happens later, delegated to TFs that appear as the neural differentiation program  
520 unfolds. Indeed, comparisons of chromatin states between stem cells and neurons support this  
521 notion. Some CNS-specific enhancers are "constitutive", i.e. accessible from the NB all the way  
522 to neurons, whereas other neuron-specific enhancers gradually become accessible at later  
523 embryonic stages [5]. A second, not mutually exclusive, scenario is that key neuronal

524 transcripts produced at the NB stage, are translationally repressed. Such genes are most likely  
525 pro-differentiation factors that generally lock cellular identity, as has been shown for the *elav*  
526 gene, whose transcription initiates in many cell types, but its protein product is strictly neuron-  
527 specific [117].

528

## 529 **CONCLUSIONS**

530 We demonstrate that during stem cell specification ASC proneurals modulate chromatin  
531 dynamics to achieve the timely activation of neural transcription. This promotes stemness but  
532 also paves the way for appropriate lineage differentiation, which may explain the onset of  
533 developmental syndromes with ASCL1 mutations (OMIM: 209880). All stem cells and their  
534 future lineages within a tissue may depend on similar mechanisms of early chromatin  
535 remodeling, which is necessary for subsequent differentiation events.

536

## 537 **METHODS**

### 538 **Drosophila stocks**

539 UAS-CD8-GFP (II); bib-Gal4 (III) homozygous females were crossed to homozygous UAS-  
540 6xmyc-scAPAA, UAS-6xmyc-l(1)sc or UAS-N $\Delta$ ecd males for the embryo collections used in  
541 ChIPseq and RNAseq experiments. The Df(1)sc-B57 and Df(1)sc<sup>19</sup> flies were rebalanced with  
542 a FM7,KrGal4,UAS-GFP chromosome to enable distinguishing the mutant embryos during  
543 imaging. Df(1)scB7/FM7,KrGal4,UAS-GFP(I); bib-Gal4(III) females were used for the UAS  
544 rescue experiments and for the UAS-FUCCI experiment.

545 For the generation of UAS-l(1)sc N-terminally 6xmyc-tagged flies, the l(1)sc coding region  
546 was amplified using primers with EcoR1 XhoI restriction sites overhangs (EcoR1-forward,  
547 XhoI-reverse) from yw cDNA (Superscript III, ThermoFisher 18080093), using KAPA High  
548 Fidelity Polymerase (Kapa/Roche, KK2103) and subsequently inserted in the entry  
549 pENTR<sup>TM</sup>3C vector (ThermoFisher, A10464). We used pTMW (Drosophila Genomics



550 Resource Center #1107) as the destination vector and the Gateway® LR Clonase® II kit  
551 (ThermoFisher, 11791020) to generate the final l(1)sc-pTMW vector. Subsequently the l(1)sc-  
552 pTMW construct was inserted into yw flies via P-element transformation . For the generation  
553 of enhancer-lacZ reporter flies we used the pBlueRabbit lacZ vector, which contains an hsp70  
554 minimal promoter upstream of a lacZ reporter gene (Housden et al. 2012). Putative proneural  
555 bound regions were amplified with the corresponding primers with overhangs for EagI (forward  
556 primers) and XbaI (reverse primers) (see Table S7) from Oregon-R genomic DNA extracted  
557 with DNAzol™ (Theromofisher). PCR fragments were extracted from agarose gels (Macherey-  
558 Nagel, 740609.250). pBlueRabbit vector was digested with EagI and XbaI, gel extracted and  
559 dephosphorylated prior to ligations. Constructs were transformed using the  $\phi$ C31 integrase  
560 system into y w nos-int ; attP40[y+] / (CyO) hosts. All vectors generated for fly transgenesis  
561 were Sanger-sequence verified (Macrogen Inc). A complete list of fly strains and primer  
562 sequences are in Additional Supplemental Methods section.

### 563 **Embryo Collections, Immunostaining and Imaging**

564 Embryo collections were made on cherry juice agar plates. Embryos were dechorionated in  
565 50% bleach for 2 minutes. Dechorionated embryos were transferred to 4 ml glass tubes  
566 containing fixative solution (1200ul 1xPBS, 800ul 10% formaldehyde, 2ml heptane) and fixed  
567 for 20min with vigorous agitation. Embryos were devitellinized by vigorous shaking in  
568 methanol for 30-40secs. After 3 quick methanol rinses, samples were stored in methanol at -  
569 20°C. On the day of immunostaining, embryos were rehydrated in PT (1xPBS, 0.2% Triton).  
570 Blocking was then conducted for at least 2 hours with PBT (PT+ 0.5% BSA). Primary  
571 antibodies were diluted in PBT and incubated overnight at 4°C. Next day, samples were washed  
572 extensively in PT. Embryos were incubated with secondary antibodies for 3 hours at room  
573 temperature. After extensive PT washes, 80 $\mu$ l n-propyl gallate-glycerol mountant was added to  
574 each sample and incubated overnight at 4°C. Embryos were then mounted and imaged in TCS  
575 SP8 confocal microscope system (Leica). Image analysis was performed with the Leica LAS  
576 X software. Antibodies used are listed in Additional Supplemental Methods section.

## 577 **ChIPseq protocol for low embryo number**

578 We developed a low input *Drosophila* embryo ChIP-seq protocol based on [118]. Briefly, we  
579 set cages of 150 homozygous UAS-CD8-GFP (II); bib-Gal4 (III) female flies with 50 males  
580 homozygous for either UAS-scAPAA (II) or UAS-l(1)sc (II), or UAS-N $\Delta$ ecd (II), pre-  
581 conditioned for two days in vials before transfer to the cages. All embryo collections were  
582 performed during the same time window, from morning to mid-afternoon, to minimize clock-  
583 mediated changes in gene expression. A 30-minute preclearing step was performed every  
584 morning of collection. Egg lays were done on cherry juice/agar 6cm dishes for 0-3 hours at  
585 27°C followed by a 3 hour maturation step at 29°C to boost GAL4 activity. We collected 3-6hs  
586 embryos on a Nitex mesh, dechorionated with 50% bleach for 2 minutes and washed with water.  
587 Subsequently, embryos were transferred with a brush in fixing solution and shaken for 10'  
588 mildly in 2ml ependorfs. Fixing solution: 1500  $\mu$ l Heptane, 100  $\mu$ l 10% FA, 200  $\mu$ l 10 X PBS  
589 and 200 double distilled H<sub>2</sub>O. Next, FA was quenched with glycine for 5' minute with mild  
590 shaking. Fixing solution was discarded and embryos were washed twice with cold 1xPBS/0.1%  
591 Triton-X and then briefly low-speed centrifuged to pellet embryos. After discarding the second  
592 PBS wash, embryo pellets were stored in -80°C. A detailed protocol can be found in Additional  
593 Supplemental Methods section.

594

## 595 ***Drosophila* Embryo RNAseq**

596 Embryos were collected at 0-2hs and then transferred to mature at 29°C for 3 hours (3-5hs  
597 collections). All embryo collections were performed during the same time window, from  
598 morning to mid-afternoon, to minimize clock-mediated changes in gene expression, after a 30-  
599 minute pre-clearing. Embryos were directly transferred in 50  $\mu$ l Trizol containing tubes and  
600 stored at -80. On the day of RNA extraction, embryos were defrosted and homogenized using  
601 1.5 ml manual pestle. For each replicate 5 independent daily collections were pooled after  
602 homogenization and RNA was isolated with phenol/chloroform without columns. RNA-seq  
603 libraries construction was performed with the Ion Total RNAseq Kit v2 (Thermo Fisher), using  
604 Poly(A) RNA selection with Dynabeads mRNA DIRECT Micro Kit Ambion (Life

605 Technologies) according to manufacturers' protocols. Libraries were sequenced on Ion  
606 Proton™ System (ThermoFisher) with PI CHIP v3, utilizing for template the Ion PI Hi-Q OT2  
607 200 kit (# A26434) and the Ion PI Hi-Q Sequencing 200 kit (# A26433, A26772).

608

#### 609 **NGS Data Analyses**

610 Fastq files were transferred from Ion Proton to IMBB servers for storage and analysis. Mapping  
611 was performed to dm6 (UCSC/dm6, iGenomes, 2015). Software and Algorithms used in this  
612 study: SAMtools [119], MACS2 (v1.4) [120], HOMER (v4.5) [121], Hisat2 [122], Cutadapt  
613 (v1.12) (doi:<https://doi.org/10.14806/ej.17.1.200>), HTSeq [123], edgeR [124], BEDTools  
614 [125], deepTools [126], GSEA (v4.0.3) [127], R (v4.0.3) (<https://www.R-project.org/>), Pavis  
615 (Flybase R6.01 assembly) [128], Flymine (v51) [63], i-cis Target [129], UCSC genome browser  
616 [130] (FlyBase/BDGP/Celera Genomics Release 6 + ISO1 MT), Flybase [64].

#### 617 **ChIPseq Peak calling, Motif Analysis and Genomic Annotation**

618 Mapping was performed using Hisat2 (--no-spliced-alignment --score-min L,0,-0.5), (samtools  
619 view -q 30). Bedgraphs were generated using bedtools genomecov and uploaded to the UCSC  
620 genome browser. Prior to peak calling, we excluded reads from the bam files mapped on  
621 repetitive regions. We also excluded reads that fell in our custom 'black list regions' (available  
622 upon request). Peak calling was performed using macs2 over input (-p 0.05) and peak overlaps  
623 were generated with bedtools (intersect -wa), excluding Chromosomes U and Uextra. The  
624 proneural consensus (Figure 1D) was generated imposing an FC>2 filter over input in the macs2  
625 output file of the stronger second replicate of scAPAA. Motif analysis was done with homer  
626 findMotifsGenome.pl --size given. Assignment of peaks to genes was performed using homer  
627 annotatePeaks.pl. The genomic distribution of the datasets was performed by homer  
628 annotatePeaks.pl dm6 (default) and Pavis with parameters of upstream and downstream length  
629 set at 5 kb.

#### 630 **Proneural Peak Consensus Overlapping with Zelda and chromatin marks during MZT**

631 We overlapped our proneural binding consensus with Zelda binding events during blastoderm  
632 cellularization (the time of the maternal to zygotic transition) from two studies [54, 56] and  
633 found 41% and 62% overlap respectively. The proneural.vs.Zelda.Harrison data overlap was a  
634 subset of the proneural.vs.Zelda.Sun therefore we decided to continue with the second,  
635 presented in Figure 1, since it gave higher overlap with the proneural cistrome. We used the  
636 Table S5 from the Harrison study and the GSE65441\_Zld\_DESeq.txt.gz from the Sun study.  
637 Both datasets were converted to Drosophila genome version dm6 from dm3 using LiftOver in  
638 the UCSC browser.

639

#### 640 **Proneural Consensus Overlaps with modENCODE datasets**

641 For the DHS st5-st14 dataset [8] we downloaded the bed files of coordinates of 5% FDR peaks  
642 from UCSC/dm3 and then used LiftOver to convert to dm6. ChIP-seq data for Ac  
643 (ENCFF073ETO), Da (ENCFF718YZD), E(spl)m8 (ENCFF074INK) were downloaded from  
644 <https://epic.gs.washington.edu/modERN/>

645

#### 646 **Heatmaps of ChIP datasets**

647 We downloaded and mapped to dm6 parameters from the following Illumina sequencing  
648 datasets: SRR1779551 (Zelda) and its input SRR1779552. NC14 histone marks SRR1505729  
649 (H3K27me3), SRR1505714 (H3K27Ac), SRR1505718 (H3K4me1) and SRR1505740 (input).  
650 SRR388356 (PolII) and SRR388382 (input). To correct for the difference in fragment size  
651 between Ion Torrent and Illumina sequencing we processed the IonTorrent datasets as follows:  
652 fastq reads were filtered and trimmed using cutadapt -m100 -l100 prior to Hisat2 mapping (--  
653 no-spliced-alignment --score-min L,0,-0.4 and samtools view -q 30). We indexed all bam files  
654 and used deepTools bamCompare, computeMatrix, plotProfile for Figure 1H and S2E and  
655 plotHeatmap to generate Figures 1G and Figure 3B. We used as reference regions the center of  
656 proneural binding events (class I and II)  $\pm 5$  kb from peak center. Heatmaps in Figures 1D and  
657 S1E were generated from the mapped reads, unprocessed for length, normalized over input,

658 using  $\pm 5$ kb from proneural peak centers, using a custom script from the Odom lab [131],  
659 exported to images by TreeView software from the Eisen lab.

660

### 661 **Boxplot of ChIP datasets**

662 For the boxplots in Fig. 3D, we used the multicov function of bedtools to count the processed  
663 trimmed reads from the H3K27Ac ChIP experiments on the stage 9 DHS dataset (Thomas et  
664 al, 2011). Subsequently, we generated the average read count per DHS from all 3 libraries and  
665 selected DHS sites that were in size equal to or greater than 50bp and had equal to or greater  
666 than 50 averaged reads per Kb of DHS. This filter resulted in 15,054 out of total 16,512 stage  
667 9 DHS sites. Of these, 2,028 exhibited proneural binding (left), while 13,026 were not bound  
668 by proneurals (middle panel). Next we normalized the read counts within each DHS over the  
669 total number of uniquely mapped reads within library to correct for library size. 1,889 DHSs  
670 exhibited at least 1.3 fold change in U-scAPAA vs U- $\Delta$ E H3K27Ac ChIP datasets (right  
671 panel). Boxplots were generated using the log<sub>2</sub> values of the corrected (for library size) reads  
672 counts in R (4.0.3). Statistics were performed with Wilcoxon rank sum tests.

673

### 674 **RNAseq Differential Analysis**

675 Mapping was performed using Hisat2 (ref, --score-min L,0,-0.5). Counts were generated from  
676 bam files with HTSeq-count (-i gene\_id). Differential Expression Analysis was performed with  
677 edgeR using batch correction and likelihood ratio tests (glmFit/glmLRT method), since  
678 replicates were performed in different time points resulting in large dispersions within groups.  
679 Tests were performed on 7,862 genes after keeping genes with cpm>3 in at least 3 samples.  
680 GSEA was performed on ranked gene lists from the edgeR output files using BDGP gene ids  
681 and genes assigned to proneural peaks or affected DHS sites.

682

### 683 **DATA ACCESS**

684 The sequencing data generated in this study have been submitted to the NCBI BioProject  
685 database (<https://www.ncbi.nlm.nih.gov/bioproject/>) under accession number PRJNA719934.

686

#### 687 **COMPETING INTEREST STATEMENT**

688 The authors declare no competing interests.

689

#### 690 **ACKNOWLEDGEMENTS**

691 We thank Ioannis Livadaras for embryo injections and Maria Monastiriotti for critical reading  
692 of the manuscript. Manolis Dialynas for data management. We thank the following students  
693 that contributed to experimental procedures: Efstathia Mpampoula, Eva Ioannou, Konstantina  
694 Mylonaki, Konstantinos Klaourakis, Krystallia Gourelia, Mary Chatzi, Christina Kosmopoulou,  
695 Christos Zioutis, Florentia Romanou and Christina Thomou. We thank Margarita Stapounzi for  
696 technical assistance. We thank Eirini Stratidaki and Niki Gounalaki at the IMBB Genomics  
697 Facility for library preparation and sequencing. We also thank Pantelis Hatzis from the  
698 Genomics Facility of Alexander Fleming Research Center Institute in Athens for library  
699 construction and sequencing one RNAseq replicate. Alexander Babaratsas for Drosophila stock  
700 maintenance.

701

#### 702 **Funding**

703 We are thankful to the Hellenic General Secretariat for Research and Innovation Postdoctoral  
704 support program (LS2. 3222) and the EU Marie Curie-CIG program (PCIG13-GA-2013-  
705 618708) for funding to VT, as well as the Fondation Sante (2017-2019) for funding to CD.

706

#### 707 **AUTHOR CONTRIBUTIONS**

708 V.T. designed, supervised, performed and analyzed the majority of experiments, wrote and  
709 prepared the manuscript. K.S., M.D., D.T. performed experiments. C.D. designed, performed  
710 experiments, analysed data and wrote the manuscript.

711

## 712 **Figure Legends**

713 **Fig. 1. Genome-wide mapping of proneural binding in *Drosophila* neuroectoderm during**  
714 **neuroblast specification.** A) Stage 9 bib-GAL4 embryo shows GAL4 activity in the cephalic  
715 and ventral neuroectoderm. B) Close ups in the neuroblast field in stage 9 embryos of the  
716 genotypes shown. C) Strategy of staged embryos used as input material to generate the ChIPseq  
717 datasets. D) Heatmaps of ChIPseq normalized signal over input centered on the proneural  
718 consensus peaks. E) Genomic snapshot at the *insc* gene. F) De novo motif analysis of the  
719 proneural consensus. G) Heatmaps of proneural, Zelda binding, histone marks and poised PolII  
720 ChIP-seq signal centered on proneural binding events, grouped in two categories: Class I  
721 occupied by Zelda earlier during MZT and Class II, Zelda-independent. H) Average of  
722 normalized ChIP-seq signal from heatmaps in G.

723 **Fig. 2. Proneurals target many genes and pathways that convey neuroblast homeostasis.**  
724 A) Gene Ontology analysis of proneural targeted genes, Biological Processes (BP), Molecular  
725 Function (MF) and Cellular Compartment (CC). B) Overlap of Flybase neuroblast genes with  
726 proneural targets shown in the 5 consecutive waves of NB specification S1-S5. Numbers under  
727 the neuroblast IDs represent the number of proneural targets over the total Flybase NB specific  
728 genes. Boxed inset lists the sum of the proneural bound neuroblast markers. C) Proneurals  
729 regulate a holistic neuroblast program. A schematic summary of selected terms. D) BDGP in  
730 situ enrichments of proneural target genes. E) A venn diagram of proneural bound genes from  
731 the BDGP database in D.

732 **Fig. 3. Proneural mediated chromatin changes correlate with transcriptional output**  
733 **during early neurogenesis.** A) A schematic representation of the strategy used to generate  
734 H3K27Ac ChIP-seq datasets and RNA-seq profiling. B) Heatmaps of H3K27Ac ChIPseq signal

735 centered on Class I and Class II proneural peaks. (C) Genomic snapshots at the *navy* and *wor*  
736 loci. \* mark DHS sites without proneural binding that exhibit increase in H3K27Ac signal  
737 in U-scAPAA vs. U-NΔE. D) Boxplots of normalized H3K27Ac signal in stage 9 DHS sites  
738 from modENCODE. DHS with proneural binding (left), not proneural-bound DHS (middle)  
739 and DHS sites that exhibited more than 30% difference in U-scAPAA versus U-NΔE conditions  
740 (right). Statistics performed with Wilcoxon rank sum tests. E) Differential Expressed Genes in  
741 scAPAA versus NΔE embryos FDR 0.2F) Gene Set Enrichment Analysis (GSEA) of RNAseq  
742 data reveal enrichment for neuroblasts and ventral epidermis. G) A venn diagram of genes with  
743 proneural binding, the affected DHS sites from E (right) and differential expressed genes  
744 ( $p < 0.05$ ) from the RNAseq. H) List of the 44 target genes from the intersection in G.

745 **Fig. 4. ASC mutant neuroblasts are temporarily stalled and devoid of stem cell identity**  
746 **markers.** A) Stage 9 wt neuroblasts (left panels) express Hb and Dpn and have divided to  
747 generate Pros positive GMCs. In *Df(1)scB57* embryos (right panels) neuroblasts express Hb  
748 but not Dpn and have not yet produced GMCs. The weak Dpn signal in the mutant embryo  
749 comes from the NE layer above the delaminated NBs. B) In stage 11 mutant neuroblasts have  
750 rebounded in Dpn expression and cell divisions to produce GMCs. The sparse Dpn and Pros  
751 positive cells outside the broad band of the VNC are PNS precursors, which are also strongly  
752 reduced in the ASC mutant. C) *Nvy-GFP* is absent in mutant neuroblasts during stage 9.  
753 Remaining expression comes from more laterally positioned PNS precursors, D) *Nvy* expression  
754 does not rebound in mutant neuroblasts at st 11. E) *Scrt* is lost or very weak in mutant  
755 neuroblasts at st 9. F) *Scrt* expression rebounds in stage 11 *Df(1)scB57* neuroblasts and GMCs.  
756

757 **Fig. 5. ASC loss is hard to compensate.** Early and late rescue phenotypes of  
758 neuroectodermally (*bibGal4*) induced proneural targets in the *Df(1)scB57* background. Early  
759 embryos (top row) stained with Dpn and Pros, late embryos (bottom row) stained with the  
760 axonal marker BP102. A) *Df(1)scB57*; *bibGal4* with no UAS transgene. B-G) as in A, plus B)  
761 UAS-*scrt* C) UAS-*wor* D) UAS-*dpn* E) UAS-*Oli* F) UAS-*ase* G) UAS-*scAPAA*. H) Model of



762 ability of selected genes to rescue the Df(1)scB57 neuronal hypoplasia I) Model of ability of  
763 selected genes to induce neuronal hyperplasia in the wt background. Activators refer to ASC  
764 genes; repressors refer to Snail and Hes family genes. Effect is shown by a check mark; lack of  
765 effect by X.

766 **Fig. 6. Proneural bound genomic elements exhibit spatiotemporal enhancer activity and**  
767 **proneural dependency.** A) Summary of enhancer spatiotemporal expression patterns in wt and  
768 Dfsc(1)scB57 (\*) embryos. B) Embryos expressing the upstream nvy-KV14 reporter. C)  
769 Embryos expressing the upstream scrt-KV10 reporter. D) Embryos expressing the upstream  
770 dpn-KV23. E) The intronic phyl-KV4 reporter in stage 10 embryos. F) The 3' prime tap-KV21  
771 reporter. G) The KV8 reporter proximal to the short brat isoforms H) The proximal to TSS insc-  
772 KV1 reporter. In the genomic insets, black arrows indicate the extent and cloning orientation  
773 of the genomic elements in the lacZ expressing vectors. The > symbol next to gene names  
774 shows the orientation of transcription.

775

## 776 REFERENCES

- 777 1. Negre N, Brown CD, Ma L, Bristow CA, Miller SW, Wagner U, Kheradpour P, Eaton  
778 ML, Loriaux P, Sealfon R, et al: **A cis-regulatory map of the Drosophila genome.**  
779 *Nature* 2011, **471**:527-531.
- 780 2. Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG,  
781 van Baren MJ, Boley N, Booth BW, et al: **The developmental transcriptome of**  
782 **Drosophila melanogaster.** *Nature* 2011, **471**:473-479.
- 783 3. Brown JB, Boley N, Eisman R, May GE, Stoiber MH, Duff MO, Booth BW, Wen J,  
784 Park S, Suzuki AM, et al: **Diversity and dynamics of the Drosophila transcriptome.**  
785 *Nature* 2014, **512**:393-399.
- 786 4. Cusanovich DA, Reddington JP, Garfield DA, Daza RM, Aghamirzaie D, Marco-  
787 Ferreres R, Pliner HA, Christiansen L, Qiu X, Steemers FJ, et al: **The cis-regulatory**

- 788 **dynamics of embryonic development at single-cell resolution.** *Nature* 2018,  
789 **555:538-542.**
- 790 5. Reddington JP, Garfield DA, Sigalova OM, Karabacak Calviello A, Marco-Ferreres R,  
791 Girardot C, Viales RR, Degner JF, Ohler U, Furlong EEM: **Lineage-Resolved**  
792 **Enhancer and Promoter Usage during a Time Course of Embryogenesis.** *Dev Cell*  
793 2020, **55:648-664 e649.**
- 794 6. mod EC, Roy S, Ernst J, Kharchenko PV, Kheradpour P, Negre N, Eaton ML, Landolin  
795 JM, Bristow CA, Ma L, et al: **Identification of functional elements and regulatory**  
796 **circuits by Drosophila modENCODE.** *Science* 2010, **330:1787-1797.**
- 797 7. Kvon EZ, Kazmar T, Stampfel G, Yanez-Cuna JO, Pagani M, Schernhuber K, Dickson  
798 BJ, Stark A: **Genome-scale functional characterization of Drosophila**  
799 **developmental enhancers in vivo.** *Nature* 2014, **512:91-95.**
- 800 8. Thomas S, Li XY, Sabo PJ, Sandstrom R, Thurman RE, Canfield TK, Giste E, Fisher  
801 W, Hammonds A, Celniker SE, et al: **Dynamic reprogramming of chromatin**  
802 **accessibility during Drosophila embryo development.** *Genome Biol* 2011, **12:R43.**
- 803 9. MacArthur S, Li XY, Li J, Brown JB, Chu HC, Zeng L, Grondona BP, Hechmer A,  
804 Simirenko L, Keranen SV, et al: **Developmental roles of 21 Drosophila transcription**  
805 **factors are determined by quantitative differences in binding to an overlapping**  
806 **set of thousands of genomic regions.** *Genome Biol* 2009, **10:R80.**
- 807 10. Zinzen RP, Girardot C, Gagneur J, Braun M, Furlong EE: **Combinatorial binding**  
808 **predicts spatio-temporal cis-regulatory activity.** *Nature* 2009, **462:65-70.**
- 809 11. Li XY, Thomas S, Sabo PJ, Eisen MB, Stamatoyannopoulos JA, Biggin MD: **The role**  
810 **of chromatin accessibility in directing the widespread, overlapping patterns of**  
811 **Drosophila transcription factor binding.** *Genome Biol* 2011, **12:R34.**
- 812 12. Spitz F, Furlong EE: **Transcription factors: from enhancer binding to**  
813 **developmental control.** *Nat Rev Genet* 2012, **13:613-626.**
- 814 13. Plank JL, Dean A: **Enhancer function: mechanistic and genome-wide insights come**  
815 **together.** *Mol Cell* 2014, **55:5-14.**

- 816 14. Hartenstein V, Wodarz A: **Initial neurogenesis in Drosophila.** *Wiley Interdiscip Rev*  
817 *Dev Biol* 2013, **2**:823.
- 818 15. Garcia-Bellido A, de Celis JF: **The complex tale of the achaete-scute complex: a**  
819 **paradigmatic case in the analysis of gene organization and function during**  
820 **development.** *Genetics* 2009, **182**:631-639.
- 821 16. Baker NE, Brown NL: **All in the family: proneural bHLH genes and neuronal**  
822 **diversity.** *Development* 2018, **145**.
- 823 17. Guillemot F, Joyner AL: **Dynamic expression of the murine Achaete-Scute**  
824 **homologue Mash-1 in the developing nervous system.** *Mech Dev* 1993, **42**:171-185.
- 825 18. Bertrand N, Castro DS, Guillemot F: **Proneural genes and the specification of neural**  
826 **cell types.** *Nat Rev Neurosci* 2002, **3**:517-530.
- 827 19. Castro DS, Martynoga B, Parras C, Ramesh V, Pacary E, Johnston C, Drechsel D,  
828 Lebel-Potter M, Garcia LG, Hunt C, et al: **A novel function of the proneural factor**  
829 **Ascl1 in progenitor proliferation identified by genome-wide characterization of**  
830 **its targets.** *Genes Dev* 2011, **25**:930-945.
- 831 20. Kageyama R, Nakanishi S: **Helix-loop-helix factors in growth and differentiation of**  
832 **the vertebrate nervous system.** *Curr Opin Genet Dev* 1997, **7**:659-665.
- 833 21. Ross SE, Greenberg ME, Stiles CD: **Basic helix-loop-helix factors in cortical**  
834 **development.** *Neuron* 2003, **39**:13-25.
- 835 22. Tepass U, Hartenstein V: **Neurogenic and proneural genes control cell fate**  
836 **specification in the Drosophila endoderm.** *Development* 1995, **121**:393-405.
- 837 23. Carmena A, Bate M, Jimenez F: **Lethal of scute, a proneural gene, participates in**  
838 **the specification of muscle progenitors during Drosophila embryogenesis.** *Genes*  
839 *Dev* 1995, **9**:2373-2383.
- 840 24. Wang CY, Shahi P, Huang JT, Phan NN, Sun Z, Lin YC, Lai MD, Werb Z: **Systematic**  
841 **analysis of the achaete-scute complex-like gene signature in clinical cancer**  
842 **patients.** *Mol Clin Oncol* 2017, **6**:7-18.

- 843 25. Ball DW: **Achaete-scute homolog-1 and Notch in lung neuroendocrine**  
844 **development and cancer.** *Cancer Lett* 2004, **204**:159-169.
- 845 26. Vias M, Massie CE, East P, Scott H, Warren A, Zhou Z, Nikitin AY, Neal DE, Mills  
846 **IG: Pro-neural transcription factors as cancer markers.** *BMC Med Genomics* 2008,  
847 **1**:17.
- 848 27. Chen H, Kunnimalaiyaan M, Van Gompel JJ: **Medullary thyroid cancer: the**  
849 **functions of raf-1 and human achaete-scute homologue-1.** *Thyroid* 2005, **15**:511-  
850 521.
- 851 28. Shida T, Furuya M, Kishimoto T, Nikaido T, Tanizawa T, Koda K, Oda K, Takano S,  
852 Kimura F, Shimizu H, et al: **The expression of NeuroD and mASH1 in the**  
853 **gastroenteropancreatic neuroendocrine tumors.** *Mod Pathol* 2008, **21**:1363-1370.
- 854 29. Somasundaram K, Reddy SP, Vinnakota K, Britto R, Subbarayan M, Nambiar S,  
855 Hebbar A, Samuel C, Shetty M, Sreepathi HK, et al: **Upregulation of ASCL1 and**  
856 **inhibition of Notch signaling pathway characterize progressive astrocytoma.**  
857 *Oncogene* 2005, **24**:7073-7083.
- 858 30. Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, Misra A, Nigro  
859 JM, Colman H, Soroceanu L, et al: **Molecular subclasses of high-grade glioma**  
860 **predict prognosis, delineate a pattern of disease progression, and resemble stages**  
861 **in neurogenesis.** *Cancer Cell* 2006, **9**:157-173.
- 862 31. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding  
863 L, Golub T, Mesirov JP, et al: **Integrated genomic analysis identifies clinically**  
864 **relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA,**  
865 **IDH1, EGFR, and NF1.** *Cancer Cell* 2010, **17**:98-110.
- 866 32. Park NI, Guilhamon P, Desai K, McAdam RF, Langille E, O'Connor M, Lan X,  
867 Whetstone H, Coutinho FJ, Vanner RJ, et al: **ASCL1 Reorganizes Chromatin to**  
868 **Direct Neuronal Fate and Suppress Tumorigenicity of Glioblastoma Stem Cells.**  
869 *Cell Stem Cell* 2017, **21**:209-224 e207.

- 870 33. Park NI, Guilhamon P, Desai K, McAdam RF, Langille E, O'Connor M, Lan X,  
871 Whetstone H, Coutinho FJ, Vanner RJ, et al: **ASCL1 Reorganizes Chromatin to**  
872 **Direct Neuronal Fate and Suppress Tumorigenicity of Glioblastoma Stem Cells.**  
873 *Cell Stem Cell* 2017, **21**:411.
- 874 34. Guillemot F, Hassan BA: **Beyond proneural: emerging functions and regulations**  
875 **of proneural proteins.** *Curr Opin Neurobiol* 2017, **42**:93-101.
- 876 35. Wapinski OL, Vierbuchen T, Qu K, Lee QY, Chanda S, Fuentes DR, Giresi PG, Ng  
877 YH, Marro S, Neff NF, et al: **Hierarchical mechanisms for direct reprogramming**  
878 **of fibroblasts to neurons.** *Cell* 2013, **155**:621-635.
- 879 36. Raposo A, Vasconcelos FF, Drechsel D, Marie C, Johnston C, Dolle D, Bithell A,  
880 Gillotin S, van den Berg DLC, Ettwiller L, et al: **Ascl1 Coordinately Regulates Gene**  
881 **Expression and the Chromatin Landscape during Neurogenesis.** *Cell Rep* 2015,  
882 **10**:1544-1556.
- 883 37. Fernandez Garcia M, Moore CD, Schulz KN, Alberto O, Donague G, Harrison MM,  
884 Zhu H, Zaret KS: **Structural Features of Transcription Factors Associating with**  
885 **Nucleosome Binding.** *Mol Cell* 2019, **75**:921-932 e926.
- 886 38. Negre B, Simpson P: **Evolution of the achaete-scute complex in insects: convergent**  
887 **duplication of proneural genes.** *Trends Genet* 2009, **25**:147-152.
- 888 39. Finet C, Decaras A, Armisen D, Khila A: **The achaete-scute complex contains a**  
889 **single gene that controls bristle development in the semi-aquatic bugs.** *Proc Biol*  
890 *Sci* 2018, **285**.
- 891 40. Hinz U, Giebel B, Campos-Ortega JA: **The basic-helix-loop-helix domain of**  
892 **Drosophila lethal of scute protein is sufficient for proneural function and activates**  
893 **neurogenic genes.** *Cell* 1994, **76**:77-87.
- 894 41. Marcellini S, Gibert JM, Simpson P: **achaete, but not scute, is dispensable for the**  
895 **peripheral nervous system of Drosophila.** *Dev Biol* 2005, **285**:545-553.

- 896 42. Cabrera CV, Martinez-Arias A, Bate M: **The expression of three members of the**  
897 **achaete-scute gene complex correlates with neuroblast segregation in Drosophila.**  
898 *Cell* 1987, **50**:425-433.
- 899 43. Skeath JB, Carroll SB: **Regulation of proneural gene expression and cell fate during**  
900 **neuroblast segregation in the Drosophila embryo.** *Development* 1992, **114**:939-946.
- 901 44. Gomez-Skarmeta JL, Campuzano S, Modolell J: **Half a century of neural**  
902 **prepatternning: the story of a few bristles and many genes.** *Nat Rev Neurosci* 2003,  
903 **4**:587-598.
- 904 45. Stern C: **Two or three bristles.** *Sci Prog (New Haven)* 1955, **Series 9**:41-84.
- 905 46. Crews ST: **Drosophila Embryonic CNS Development: Neurogenesis, Gliogenesis,**  
906 **Cell Fate, and Differentiation.** *Genetics* 2019, **213**:1111-1144.
- 907 47. Jimenez F, Campos-Ortega JA: **Defective neuroblast commitment in mutants of the**  
908 **achaete-scute complex and adjacent genes of D. melanogaster.** *Neuron* 1990, **5**:81-  
909 89.
- 910 48. Kiparaki M, Zarifi I, Delidakis C: **bHLH proteins involved in Drosophila**  
911 **neurogenesis are mutually regulated at the level of stability.** *Nucleic Acids Res*  
912 2015, **43**:2543-2559.
- 913 49. Fuerstenberg S, Giniger E: **Multiple roles for notch in Drosophila myogenesis.** *Dev*  
914 *Biol* 1998, **201**:66-77.
- 915 50. Kudron MM, Victorsen A, Gevirtzman L, Hillier LW, Fisher WW, Vafeados D, Kirkey  
916 M, Hammonds AS, Gersch J, Ammouri H, et al: **The ModERN Resource: Genome-**  
917 **Wide Binding Profiles for Hundreds of Drosophila and Caenorhabditis elegans**  
918 **Transcription Factors.** *Genetics* 2018, **208**:937-949.
- 919 51. Southall TD, Brand AH: **Neural stem cell transcriptional networks highlight genes**  
920 **essential for nervous system development.** *EMBO J* 2009, **28**:3799-3807.
- 921 52. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN,  
922 Hauschka SD, Lassar AB, et al.: **Interactions between heterologous helix-loop-helix**

- 923            **proteins generate complexes that bind specifically to a common DNA sequence.**
- 924            *Cell* 1989, **58**:537-544.
- 925    53.    Liang HL, Nien CY, Liu HY, Metzstein MM, Kirov N, Rushlow C: **The zinc-finger**
- 926            **protein Zelda is a key activator of the early zygotic genome in Drosophila.** *Nature*
- 927            2008, **456**:400-403.
- 928    54.    Harrison MM, Li XY, Kaplan T, Botchan MR, Eisen MB: **Zelda binding in the early**
- 929            **Drosophila melanogaster embryo marks regions subsequently activated at the**
- 930            **maternal-to-zygotic transition.** *PLoS Genet* 2011, **7**:e1002266.
- 931    55.    Li XY, Harrison MM, Villalta JE, Kaplan T, Eisen MB: **Establishment of regions of**
- 932            **genomic activity during the Drosophila maternal to zygotic transition.** *Elife* 2014,
- 933            **3.**
- 934    56.    Sun Y, Nien CY, Chen K, Liu HY, Johnston J, Zeitlinger J, Rushlow C: **Zelda**
- 935            **overcomes the high intrinsic nucleosome barrier at enhancers during Drosophila**
- 936            **zygotic genome activation.** *Genome Res* 2015, **25**:1703-1714.
- 937    57.    Xu Z, Chen H, Ling J, Yu D, Struffi P, Small S: **Impacts of the ubiquitous factor**
- 938            **Zelda on Bicoid-dependent DNA binding and transcription in Drosophila.** *Genes*
- 939            *Dev* 2014, **28**:608-621.
- 940    58.    Schulz KN, Bondra ER, Moshe A, Villalta JE, Lieb JD, Kaplan T, McKay DJ, Harrison
- 941            MM: **Zelda is differentially required for chromatin accessibility, transcription**
- 942            **factor binding, and gene expression in the early Drosophila embryo.** *Genome Res*
- 943            2015, **25**:1715-1726.
- 944    59.    Hug CB, Grimaldi AG, Kruse K, Vaquerizas JM: **Chromatin Architecture Emerges**
- 945            **during Zygotic Genome Activation Independent of Transcription.** *Cell* 2017,
- 946            **169**:216-228 e219.
- 947    60.    Gaertner B, Zeitlinger J: **RNA polymerase II pausing during development.**
- 948            *Development* 2014, **141**:1179-1183.

- 949 61. Zeitlinger J, Stark A, Kellis M, Hong JW, Nechaev S, Adelman K, Levine M, Young  
950 RA: **RNA polymerase stalling at developmental control genes in the *Drosophila***  
951 ***melanogaster* embryo.** *Nat Genet* 2007, **39**:1512-1516.
- 952 62. Lagha M, Bothma JP, Esposito E, Ng S, Stefanik L, Tsui C, Johnston J, Chen K,  
953 Gilmour DS, Zeitlinger J, Levine MS: **Paused Pol II coordinates tissue**  
954 **morphogenesis in the *Drosophila* embryo.** *Cell* 2013, **153**:976-987.
- 955 63. Lyne R, Smith R, Rutherford K, Wakeling M, Varley A, Guillier F, Janssens H, Ji W,  
956 McLaren P, North P, et al: **FlyMine: an integrated database for *Drosophila* and**  
957 ***Anopheles* genomics.** *Genome Biol* 2007, **8**:R129.
- 958 64. Larkin A, Marygold SJ, Antonazzo G, Attrill H, Dos Santos G, Garapati PV, Goodman  
959 JL, Gramates LS, Millburn G, Strelets VB, et al: **FlyBase: updates to the *Drosophila***  
960 ***melanogaster* knowledge base.** *Nucleic Acids Res* 2021, **49**:D899-D907.
- 961 65. Egger B, Boone JQ, Stevens NR, Brand AH, Doe CQ: **Regulation of spindle**  
962 **orientation and neural stem cell fate in the *Drosophila* optic lobe.** *Neural Dev* 2007,  
963 **2**:1.
- 964 66. Isshiki T, Pearson B, Holbrook S, Doe CQ: ***Drosophila* neuroblasts sequentially**  
965 **express transcription factors which specify the temporal identity of their neuronal**  
966 **progeny.** *Cell* 2001, **106**:511-521.
- 967 67. Simoes S, Oh Y, Wang MFZ, Fernandez-Gonzalez R, Tepass U: **Myosin II promotes**  
968 **the anisotropic loss of the apical domain during *Drosophila* neuroblast ingression.**  
969 *J Cell Biol* 2017, **216**:1387-1404.
- 970 68. Sousa-Nunes R, Somers WG: **Mechanisms of asymmetric progenitor divisions in**  
971 **the *Drosophila* central nervous system.** *Adv Exp Med Biol* 2013, **786**:79-102.
- 972 69. Feinstein PG, Kornfeld K, Hogness DS, Mann RS: **Identification of homeotic target**  
973 **genes in *Drosophila melanogaster* including *nervy*, a proto-oncogene homologue.**  
974 *Genetics* 1995, **140**:573-586.



- 975 70. Ashraf SI, Hu X, Roote J, Ip YT: **The mesoderm determinant snail collaborates with**  
976 **related zinc-finger proteins to control Drosophila neurogenesis.** *EMBO J* 1999,  
977 **18:6426-6438.**
- 978 71. Zielke N, Korzelius J, van Straaten M, Bender K, Schuhknecht GFP, Dutta D, Xiang  
979 J, Edgar BA: **Fly-FUCCI: A versatile tool for studying cell proliferation in complex**  
980 **tissues.** *Cell Rep* 2014, **7:588-598.**
- 981 72. Martin-Bermudo MD, Gonzalez F, Dominguez M, Rodriguez I, Ruiz-Gomez M,  
982 Romani S, Modolell J, Jimenez F: **Molecular characterization of the lethal of scute**  
983 **genetic function.** *Development* 1993, **118:1003-1012.**
- 984 73. Arzan Zarin A, Labrador JP: **Motor axon guidance in Drosophila.** *Semin Cell Dev*  
985 *Biol* 2019, **85:36-47.**
- 986 74. von Hilchen CM, Beckervordersandforth RM, Rickert C, Technau GM, Altenhein B:  
987 **Identity, origin, and migration of peripheral glial cells in the Drosophila embryo.**  
988 *Mech Dev* 2008, **125:337-352.**
- 989 75. Howard LJ, Brown HE, Wadsworth BC, Evans TA: **Midline axon guidance in the**  
990 **Drosophila embryonic central nervous system.** *Semin Cell Dev Biol* 2019, **85:13-25.**
- 991 76. Sepp KJ, Auld VJ: **Reciprocal interactions between neurons and glia are required**  
992 **for Drosophila peripheral nervous system development.** *J Neurosci* 2003, **23:8221-**  
993 **8230.**
- 994 77. Araujo SJ, Tear G: **Axon guidance mechanisms and molecules: lessons from**  
995 **invertebrates.** *Nat Rev Neurosci* 2003, **4:910-922.**
- 996 78. Griffiths RL, Hidalgo A: **Prospero maintains the mitotic potential of glial**  
997 **precursors enabling them to respond to neurons.** *EMBO J* 2004, **23:2440-2450.**
- 998 79. Hosoya T, Takizawa K, Nitta K, Hotta Y: **glial cells missing: a binary switch between**  
999 **neuronal and glial determination in Drosophila.** *Cell* 1995, **82:1025-1036.**
- 1000 80. Skeath JB, Doe CQ: **The achaete-scute complex proneural genes contribute to**  
1001 **neural precursor specification in the Drosophila CNS.** *Curr Biol* 1996, **6:1146-**  
1002 **1152.**

- 1003 81. Parras C, Garcia-Alonso LA, Rodriguez I, Jimenez F: **Control of neural precursor**  
1004 **specification by proneural proteins in the CNS of Drosophila.** *EMBO J* 1996,  
1005 **15:6394-6399.**
- 1006 82. Grenningloh G, Rehm EJ, Goodman CS: **Genetic analysis of growth cone guidance**  
1007 **in Drosophila: fasciclin II functions as a neuronal recognition molecule.** *Cell* 1991,  
1008 **67:45-57.**
- 1009 83. Higashijima S, Shishido E, Matsuzaki M, Saigo K: **eagle, a member of the steroid**  
1010 **receptor gene superfamily, is expressed in a subset of neuroblasts and regulates**  
1011 **the fate of their putative progeny in the Drosophila CNS.** *Development* 1996,  
1012 **122:527-536.**
- 1013 84. Nieto MA: **The snail superfamily of zinc-finger transcription factors.** *Nat Rev Mol*  
1014 *Cell Biol* 2002, **3:155-166.**
- 1015 85. Kageyama R, Ohtsuka T, Kobayashi T: **The Hes gene family: repressors and**  
1016 **oscillators that orchestrate embryogenesis.** *Development* 2007, **134:1243-1251.**
- 1017 86. Bahrapour S, Gunnar E, Jonsson C, Ekman H, Thor S: **Neural Lineage Progression**  
1018 **Controlled by a Temporal Proliferation Program.** *Dev Cell* 2017, **43:332-348 e334.**
- 1019 87. Homem CC, Knoblich JA: **Drosophila neuroblasts: a model for stem cell biology.**  
1020 *Development* 2012, **139:4297-4310.**
- 1021 88. Pi H, Huang SK, Tang CY, Sun YH, Chien CT: **phyllopod is a target gene of**  
1022 **proneural proteins in Drosophila external sensory organ development.** *Proc Natl*  
1023 *Acad Sci U S A* 2004, **101:8378-8383.**
- 1024 89. Bodmer R, Carretto R, Jan YN: **Neurogenesis of the peripheral nervous system in**  
1025 **Drosophila embryos: DNA replication patterns and cell lineages.** *Neuron* 1989,  
1026 **3:21-32.**
- 1027 90. Jarman AP, Groves AK: **The role of Atonal transcription factors in the**  
1028 **development of mechanosensitive cells.** *Semin Cell Dev Biol* 2013, **24:438-447.**

- 1029 91. Huang ML, Hsu CH, Chien CT: **The proneural gene amos promotes multiple**  
1030 **dendritic neuron formation in the Drosophila peripheral nervous system.** *Neuron*  
1031 2000, **25**:57-67.
- 1032 92. Thurman RE, Rynes E, Humbert R, Vierstra J, Maurano MT, Haugen E, Sheffield NC,  
1033 Stergachis AB, Wang H, Vernot B, et al: **The accessible chromatin landscape of the**  
1034 **human genome.** *Nature* 2012, **489**:75-82.
- 1035 93. Neph S, Vierstra J, Stergachis AB, Reynolds AP, Haugen E, Vernot B, Thurman RE,  
1036 John S, Sandstrom R, Johnson AK, et al: **An expansive human regulatory lexicon**  
1037 **encoded in transcription factor footprints.** *Nature* 2012, **489**:83-90.
- 1038 94. Stergachis AB, Neph S, Reynolds A, Humbert R, Miller B, Paige SL, Vernot B, Cheng  
1039 JB, Thurman RE, Sandstrom R, et al: **Developmental fate and cellular maturity**  
1040 **encoded in human regulatory DNA landscapes.** *Cell* 2013, **154**:888-903.
- 1041 95. Argelaguet R, Clark SJ, Mohammed H, Stapel LC, Krueger C, Kapourani CA, Imaz-  
1042 Rosshandler I, Lohoff T, Xiang Y, Hanna CW, et al: **Multi-omics profiling of mouse**  
1043 **gastrulation at single-cell resolution.** *Nature* 2019, **576**:487-491.
- 1044 96. Long HK, Prescott SL, Wysocka J: **Ever-Changing Landscapes: Transcriptional**  
1045 **Enhancers in Development and Evolution.** *Cell* 2016, **167**:1170-1187.
- 1046 97. Marshall OJ, Brand AH: **Chromatin state changes during neural development**  
1047 **revealed by in vivo cell-type specific profiling.** *Nat Commun* 2017, **8**:2271.
- 1048 98. Abdusselamoglu MD, Landskron L, Bowman SK, Eroglu E, Burkard T, Kingston RE,  
1049 Knoblich JA: **Dynamics of activating and repressive histone modifications in**  
1050 **Drosophila neural stem cell lineages and brain tumors.** *Development* 2019, **146**.
- 1051 99. Zenk F, Loeser E, Schiavo R, Kilpert F, Bogdanovic O, Iovino N: **Germ line-inherited**  
1052 **H3K27me3 restricts enhancer function during maternal-to-zygotic transition.**  
1053 *Science* 2017, **357**:212-216.
- 1054 100. Foo SM, Sun Y, Lim B, Ziukaite R, O'Brien K, Nien CY, Kirov N, Shvartsman SY,  
1055 Rushlow CA: **Zelda potentiates morphogen activity by increasing chromatin**  
1056 **accessibility.** *Curr Biol* 2014, **24**:1341-1346.

- 1057 101. ten Bosch JR, Benavides JA, Cline TW: **The TAGteam DNA motif controls the**  
1058 **timing of Drosophila pre-blastoderm transcription.** *Development* 2006, **133**:1967-  
1059 1977.
- 1060 102. Karaiskos N, Wahle P, Alles J, Boltengagen A, Ayoub S, Kipar C, Kocks C, Rajewsky  
1061 N, Zinzen RP: **The Drosophila embryo at single-cell transcriptome resolution.**  
1062 *Science* 2017, **358**:194-199.
- 1063 103. Pattyn A, Guillemot F, Brunet JF: **Delays in neuronal differentiation in Mash1/Ascl1**  
1064 **mutants.** *Dev Biol* 2006, **295**:67-75.
- 1065 104. Arefin B, Parvin F, Bahrapour S, Stadler CB, Thor S: **Drosophila Neuroblast**  
1066 **Selection Is Gated by Notch, Snail, SoxB, and EMT Gene Interplay.** *Cell Rep* 2019,  
1067 **29**:3636-3651 e3633.
- 1068 105. Ashraf SI, Ip YT: **The Snail protein family regulates neuroblast expression of**  
1069 **inscuteable and string, genes involved in asymmetry and cell division in**  
1070 **Drosophila.** *Development* 2001, **128**:4757-4767.
- 1071 106. Jafar-Nejad H, Tien AC, Acar M, Bellen HJ: **Senseless and Daughterless confer**  
1072 **neuronal identity to epithelial cells in the Drosophila wing margin.** *Development*  
1073 2006, **133**:1683-1692.
- 1074 107. Jarman AP, Brand M, Jan LY, Jan YN: **The regulation and function of the helix-**  
1075 **loop-helix gene, asense, in Drosophila neural precursors.** *Development* 1993,  
1076 **119**:19-29.
- 1077 108. Stampfel G, Kazmar T, Frank O, Wienerroither S, Reiter F, Stark A: **Transcriptional**  
1078 **regulators form diverse groups with context-dependent regulatory functions.**  
1079 *Nature* 2015, **528**:147-151.
- 1080 109. Cai Y, Chia W, Yang X: **A family of snail-related zinc finger proteins regulates two**  
1081 **distinct and parallel mechanisms that mediate Drosophila neuroblast asymmetric**  
1082 **divisions.** *EMBO J* 2001, **20**:1704-1714.

- 1083 110. Lai SL, Miller MR, Robinson KJ, Doe CQ: **The Snail family member Worniu is**  
1084 **continuously required in neuroblasts to prevent Elav-induced premature**  
1085 **differentiation.** *Dev Cell* 2012, **23**:849-857.
- 1086 111. Stagg SB, Guardiola AR, Crews ST: **Dual role for Drosophila lethal of scute in CNS**  
1087 **midline precursor formation and dopaminergic neuron and motoneuron cell fate.**  
1088 *Development* 2011, **138**:2171-2183.
- 1089 112. Jarman AP, Ahmed I: **The specificity of proneural genes in determining Drosophila**  
1090 **sense organ identity.** *Mech Dev* 1998, **76**:117-125.
- 1091 113. Chien CT, Hsiao CD, Jan LY, Jan YN: **Neuronal type information encoded in the**  
1092 **basic-helix-loop-helix domain of proneural genes.** *Proc Natl Acad Sci U S A* 1996,  
1093 **93**:13239-13244.
- 1094 114. Nieto M, Schuurmans C, Britz O, Guillemot F: **Neural bHLH genes control the**  
1095 **neuronal versus glial fate decision in cortical progenitors.** *Neuron* 2001, **29**:401-  
1096 413.
- 1097 115. Tomita K, Moriyoshi K, Nakanishi S, Guillemot F, Kageyama R: **Mammalian**  
1098 **achaete-scute and atonal homologs regulate neuronal versus glial fate**  
1099 **determination in the central nervous system.** *EMBO J* 2000, **19**:5460-5472.
- 1100 116. Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg  
1101 ME: **Neurogenin promotes neurogenesis and inhibits glial differentiation by**  
1102 **independent mechanisms.** *Cell* 2001, **104**:365-376.
- 1103 117. Sanfilippo P, Smibert P, Duan H, Lai EC: **Neural specificity of the RNA-binding**  
1104 **protein Elav is achieved by post-transcriptional repression in non-neural tissues.**  
1105 *Development* 2016, **143**:4474-4485.
- 1106 118. Schmidt D, Wilson MD, Spyrou C, Brown GD, Hadfield J, Odom DT: **ChIP-seq:**  
1107 **using high-throughput sequencing to discover protein-DNA interactions.** *Methods*  
1108 2009, **48**:240-248.

- 1109 119. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G,  
1110 Durbin R, Genome Project Data Processing S: **The Sequence Alignment/Map format**  
1111 **and SAMtools**. *Bioinformatics* 2009, **25**:2078-2079.
- 1112 120. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nusbaum C,  
1113 Myers RM, Brown M, Li W, Liu XS: **Model-based analysis of ChIP-Seq (MACS)**.  
1114 *Genome Biol* 2008, **9**:R137.
- 1115 121. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh  
1116 H, Glass CK: **Simple combinations of lineage-determining transcription factors**  
1117 **prime cis-regulatory elements required for macrophage and B cell identities**. *Mol*  
1118 *Cell* 2010, **38**:576-589.
- 1119 122. Kim D, Langmead B, Salzberg SL: **HISAT: a fast spliced aligner with low memory**  
1120 **requirements**. *Nat Methods* 2015, **12**:357-360.
- 1121 123. Anders S, Pyl PT, Huber W: **HTSeq--a Python framework to work with high-**  
1122 **throughput sequencing data**. *Bioinformatics* 2015, **31**:166-169.
- 1123 124. Robinson MD, McCarthy DJ, Smyth GK: **edgeR: a Bioconductor package for**  
1124 **differential expression analysis of digital gene expression data**. *Bioinformatics*  
1125 2010, **26**:139-140.
- 1126 125. Quinlan AR, Hall IM: **BEDTools: a flexible suite of utilities for comparing genomic**  
1127 **features**. *Bioinformatics* 2010, **26**:841-842.
- 1128 126. Ramirez F, Ryan DP, Gruning B, Bhardwaj V, Kilpert F, Richter AS, Heyne S, Dundar  
1129 F, Manke T: **deepTools2: a next generation web server for deep-sequencing data**  
1130 **analysis**. *Nucleic Acids Res* 2016, **44**:W160-165.
- 1131 127. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA,  
1132 Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: **Gene set enrichment**  
1133 **analysis: a knowledge-based approach for interpreting genome-wide expression**  
1134 **profiles**. *Proc Natl Acad Sci U S A* 2005, **102**:15545-15550.
- 1135 128. Huang W, Loganantharaj R, Schroeder B, Fargo D, Li L: **PAVIS: a tool for Peak**  
1136 **Annotation and Visualization**. *Bioinformatics* 2013, **29**:3097-3099.

- 1137 129. Herrmann C, Van de Sande B, Potier D, Aerts S: **i-cisTarget: an integrative genomics**  
1138 **method for the prediction of regulatory features and cis-regulatory modules.**  
1139 *Nucleic Acids Res* 2012, **40**:e114.
- 1140 130. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D:  
1141 **The human genome browser at UCSC.** *Genome Res* 2002, **12**:996-1006.
- 1142 131. Schmidt D, Schwalie PC, Wilson MD, Ballester B, Goncalves A, Kutter C, Brown GD,  
1143 Marshall A, Flicek P, Odom DT: **Waves of retrotransposon expansion remodel**  
1144 **genome organization and CTCF binding in multiple mammalian lineages.** *Cell*  
1145 2012, **148**:335-348.
- 1146
- 1147
- 1148
- 1149
- 1150
- 1151
- 1152
- 1153
- 1154
- 1155
- 1156
- 1157
- 1158
- 1159

1160