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ORIGINAL ARTICLE

Expression of Mammalian BM88/CEND1 in *Drosophila* Affects Nervous System Development by Interfering with Precursor Cell Formation

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Abstract We used *Drosophila melanogaster* as an experimental model to express mouse and pig BM88/CEND1 (cell cycle exit and neuronal differentiation 1) in order to investigate its potential functional effects on *Drosophila* neurogenesis. BM88/CEND1 is a neuron-specific protein whose function is implicated in triggering cells to exit from the cell cycle and differentiate towards a neuronal phenotype. Transgenic flies expressing either mouse or pig BM88/CEND1 in the nervous system had severe neuronal phenotypes with variable expressivity at various stages of embryonic development. In early embryonic stage 10, BM88/CEND1 expression led to an increase in the neural-specific antigenicity of neuroectoderm at the expense of precursor cells [neuroblasts (Nbs) and ganglion mother cells (GMCs)] including the defective formation and differentiation of the MP2 precursors, whereas at later stages (12–15), protein accumulation induced gross morphological defects primarily in the CNS accompanied by a reduction of Nb and GMC markers. Furthermore, the neuronal precursor cells of embryos expressing BM88/CEND1 failed to carry out proper cell-cycle progression as revealed by the disorganized expression patterns of specific cell-cycle markers. BM88/CEND1 accumulation in the *Drosophila* eye affected normal eye disc development by disrupting the ommatidia. Finally, we demonstrated that expression of BM88/CEND1 modified/reduced the levels of activated MAP kinase indicating a functional effect of BM88/CEND1 on the MAPK signaling pathway. Our findings suggest that the expression of mammalian BM88/CEND1 in *Drosophila* exerts specific functional effects associated with neuronal precursor cell formation during embryonic neurogenesis and proper eye disc development. This study also validates the use of *Drosophila* as a powerful model system in which to investigate gene function and the underlying molecular mechanisms.

Keywords Drosophila · Neurogenesis · BM88/CEND1 · Nervous system · Protein function

Introduction

The central nervous systems (CNS) of *Drosophila* and vertebrates, although evolutionarily separated, share remarkable similarities in both neurons and glia, using the same neurotransmitters and possessing conserved basic molecular mechanisms during neural development. Comparable mechanisms provide positional information for patterning the CNS along the dorsoventral and anteriposterior body axes. Genetic pathways and molecular mechanisms conserved between *Drosophila* and vertebrates regulate neuronal precursor formation, cell fate specification, and proper formation of the nervous system [1, 2]. These similar molecular characteristics, in combination with the great variety of established genetic tools, render *Drosophila* a useful model organism in which to study

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gene function and the associated molecular mechanisms underlying human disease [2–5].

Because of its relative simplicity, the *Drosophila* embryonic nervous system offers a key to elucidating the molecular determinants and understanding the regulatory mechanisms essential for nervous system development. The *Drosophila* embryonic CNS consisting of the central brain and the ventral nerve cord (VNC) develops from the neuroectoderm that lies on either side of a narrow strip of ventral midline cells [6–8]. Early in embryogenesis, patterning genes acting along the dorsoventral and anterioposterior axes subdivide the neuroectoderm into a fixed, segmented pattern of neural equivalence groups (proneural clusters). Neuroblasts (Nbs) are first formed during embryonic stages 9 to 11 as single cells expressing the highest levels of *achaete-scute* (ac/sc) protein complex. Nbs delaminate from the embryonic neuroepithelium (surface) and move into the interior of the embryo. Embryonic Nbs are specified in a process called lateral inhibition in which Notch/Delta signaling refines the expression of proneural genes to individual cells, extinguishing ac/sc expression from the remaining cells of the cluster which, in turn, remain undifferentiated or undergo epidermal differentiation [6–9]. Shortly after specification/delamination, Nbs undergo repeated self-renewing asymmetric divisions, each giving rise to another Nb and a smaller ganglion mother cell (GMC). A key step in this process involves segregation of the Prospero protein into the GMC, where it resides in the cell cortex. Prospero rapidly translocates to the nucleus where it represses cell-cycle gene expression thereby inhibiting the proliferative potential of GMCs. Each GMC then divides once to generate two post-mitotic neurons and/or glia [9–11].

During neurogenesis in *Drosophila*, most Nbs belong to the type I class producing neuronal lineages through non-self-renewing GMCs, generating all VNC neurons and most of the central brain in the embryo. A small subset of brain Nbs belonging to the type II class produce exceptionally large neuronal lineages through self-renewing intermediate progenitor cells, each producing 4–6 GMCs and 8–12 neurons (type II lineage), generating clones of up to 500 cells giving rise to central brain neurons [12]. Embryonic Nb divisions produce all the neurons that form the larval CNS but only 10% of the cells in the adult CNS. In the abdominal regions of the embryo, most Nbs are eliminated through programmed cell death after completing their neuronal lineages. In the cephalic and thoracic regions, however, Nbs arrest their cell cycle and exit from G1 into a G0-like quiescent state until larval stages. At late L1/early L2 stages, about 100 Nbs start dividing again (second wave of neurogenesis) and generate the remaining 90% of adult neurons. Neurogenesis continues throughout larval into pupal stages, at which point the Nbs exit from the cell cycle and disappear [10, 12–16].

Several studies in *Drosophila* have identified a number of genes whose products, like Prospero, play key roles during neurogenesis especially in Nb development, asymmetric division, and differentiation. Some of these include the ac/sc protein complex, *single minded*, Numb, Partner of Numb, Miranda, Inscutable, *Drosophila* TNF-receptor-associated factor, polo, Aurora, and Notch [6, 8, 11, 16–21]. Most of these genes participate in master regulatory cascades which include the Notch- and/or epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK)-dependent signaling pathways [22–25]. Both evolutionarily conserved pathways provide instructive signals and unique spatio-temporal regulation during *Drosophila* development. Differential activation of EGFR/MAPK signaling controls patterning of the VNC as well as the formation and specification of Nbs within the developing embryonic CNS. In EGFR-mutant embryos, lateral cell fates replace ventral cell fates leading to gross disruption of the CNS [22, 26–28]. Activation of EGFR signaling in a single-burst mode is responsible for proper patterning of the ventral embryonic ectoderm whereas multiple cycles of EGFR activation are required to maintain cell fate, viability, and normal development in the *Drosophila* eye disc [29, 30]. In many developmental contexts, EGFR signaling communicates with the proneural/Notch signaling pathway to mediate finer and more precise regulation of developmental processes [25, 28–32].

In addition to genes and molecular pathways highly-conserved between flies and mammals mediating neurogenesis, information has emerged regarding genes critical for mammalian nervous system development without apparent evolutionary counterparts in *Drosophila*. One such gene product is BM88/CEND1 (cell cycle exit and neuronal differentiation 1), encoding a neuronal-specific protein whose expression correlates with neuronal birth, the progression of progenitor cells towards neuronal differentiation, and terminal differentiation itself [33–36]. BM88/CEND1 protein is a homodimer of ~140 amino-acids, depending on the species, and possesses a putative transmembrane domain at the C terminus. Homologous proteins have been identified only in vertebrates including mouse, rat, chicken, and human [35, 37, 38]. Taxonomically, vertebrates are part of the subphylum Vertebrata/Craniata and belong to the phylum Chordata, in the superphylum Deuterostomia which, in turn, belongs to the clades Nephrozoa and Bilateria (members of the subkingdom Eumetazoa). Sequence analysis of BM88/CEND1 using bioinformatics database tools from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), EXPASY (UniProtKB, PROSITE, www.expasy.org), and the European Bioinformatics Institute

(EMBL-EBI, www.ebi.ac.uk) has revealed that, at least in terms of sequence homology, BM88/CEND1 orthologs appear to be present only in vertebrate species showing > 65% sequence identity (Tzortzopoulos and Skoulakis, unpublished observations). Nevertheless, the existence of a more divergent ortholog in *Drosophila* cannot be excluded at this point and remains to be elucidated.

Furthermore, the exact molecular action of BM88/CEND1 protein has not yet been fully uncovered. Studies have shown that excessive protein accumulation in mouse neuroblastoma Neuro 2a (N2A) cells is sufficient to drive them towards exit from the cell-cycle, promoting neuronal differentiation in the absence of external stimuli [39]. BM88 expression is downregulated and accompanied by loss of retinal ganglion cells after optic nerve crush and complete optic nerve transection in young adult rats [40]. BM88 has been proposed to be an early marker of proliferating neuronal precursor cells [36] and also an indicator of abnormal functioning of retinal ganglion cells, preceding cell death [40]. Combination of gain- and loss-of-function approaches in N2A cells and chick embryos has demonstrated that BM88/CEND1 has dual functional effects. It is necessary and sufficient for cell-cycle exit and neuronal differentiation by controlling the balance between cell proliferation and cell-cycle exit [41, 42]. It has also been reported that BM88 plays a role in neuronal differentiation by releasing the Notch inhibition of proneural genes [43]. Similarly, more recently, a pivotal role of BM88/CEND1 in coordinating cell-cycle exit and the differentiation of neuronal precursors has been demonstrated in a Ran binding protein- and dual-specificity tyrosine phosphorylation-regulated kinase 1A-dependent manner [44] in mouse neuroblastoma Neuro 2a cells as well as in an Ahi1 ((Abelson helper integration site 1))-dependent manner in mice [45].

In this study, we investigated the ability of BM88/CEND1 to mediate specific neuronal functional effects using *Drosophila* as a model system and as a species without an identified, apparent BM88/CEND1 ortholog. We aimed to determine whether ectopic BM88/CEND1 expression would respond to context-specific stimuli and thus interfere with neurogenesis in *Drosophila*, indicating engagement of evolutionarily-conserved molecular pathways. We found that it specifically interfered with normal development of the embryonic nervous system, affecting the formation of neuronal precursors. BM88/CEND1 expression also affected normal eye disc development by disrupting the stereotyped external ommatidia morphology and the internal eye disc structure. Our findings suggest that mouse and pig BM88/CEND1 proteins are functional in *Drosophila* and generate deficits in normal neurogenesis, providing also experimental evidence that BM88/CEND1 functionally interferes with molecular constituents of the conserved MAPK signaling pathway.

Experimental Procedures

Fly Strains

The y^1/w^{67c23} strain was provided by Prof. C. Delidakis, while the w^{1118} control flies have been described by Acevedo *et al.* [46]. *achaete-GAL4*, $elav^{c155}$*-GAL4*, *mef2-GAL4*, *twist;mef2-GAL4*, *GMR-GAL4*, *sevenless*-GAL4, and *c772-GAL4* were obtained from the Bloomington Drosophila Stock Centre (Bloomington, USA). Flies were cultured on a standard wheat-flour-sugar food supplemented with soy flour and $CaCl_2$. Crosses were carried out at 27°C unless otherwise stated.

Generation of UAS-Pig and Mouse BM88/CEND1 Lines

The UAS-pBM88/CEND1 and mBM88/CEND1 lines were constructed by cloning the full-length pig and mouse cDNAs each into pUAST [47]. Cloning was verified by sequencing. Embryo injections and P-element-mediated transformation producing transgenic flies were performed according to standard procedures [48]. Five independent transformant lines were obtained for UAS-pBM88/CEND1 and four for UAS-mBM88/CEND1. All independent lines were characterized phenotypically by expressing the transgene under the control of the pan-neuronal $elav^{c155}$ driver. The phenotype was classified based on its severity and we present results from the two strongest independent UAS-pig and mouse BM88/CEND1 transformant strains.

Antibodies and Immunohistochemistry

Embryos were collected, dechorionated, fixed, immunostained, and mounted according to previously-published protocols [49, 50]. Horizontal and frontal adult brain sections (5 μm thick) were cut, fixed, and paraffinized according to standard procedures [46, 51]. The following primary antibodies were used: rabbit anti-horseradish peroxidase (HRP) (1:500), mouse anti-Elav (1:10), mouse anti-BP104 (1:10), mouse anti-Prospero (1:10), mouse anti-cyclin A (from the Developmental Studies Hybridoma Bank, Iowa city, Iowa), mouse anti-Worniu (1:1000) kindly provided by Dr. X. Yang [52], rabbit anti-Phosphohistone 3 (PH3) (1:500), mouse anti-MAPK (1:50) (Sigma), rabbit polyclonal and mouse monoclonal anti-BM88/CEND1 [42], and rabbit anti-leo (1:4000) [51]. Biotinylated secondary antibodies (Jackson Immunochemicals, West Grove, USA) and secondary antibodies

conjugated to Alexa Fluor 488 or 555 (Molecular Probes) were used at 1:2000.

Western Blot Analysis

Western blots of total protein extracts of embryos derived from *elav*^{c155}-GAL4>UAS-mBM88/CEND1 or *elav*^{c155}-GAL4>UAS-pBM88/CEND1 and *achaete*-GAL4>UAS-mBM88/CEND1 or *achaete-GAL4*>UAS-pBM88/CEND1 females collected overnight were probed using rabbit polyclonal BM88/CEND1 antibody diluted 1:400.

Reverse Transcription-PCR

Drosophila embryos (100 per genotype) were collected at the desired developmental stage (4 h and 10 h), RNA was extracted, and cDNA was prepared as previously described [53] and rapidly dissolved in TRIzol. Seven hundred nanograms of total RNA was used per reverse transcription. PCR primers used were: 5′-GCCAAGGCTACCACTGA and 5′-CAGGAAAACCACACCGAGA for *pBM88/CEND1*, and 5′-GATCGTGAAGAAGCGCAC and 5′-CGCTCGACAATCTCCTTG for *rp49*. Detection of *rp49* transcripts was used to monitor the quality of the RT-PCR reactions and served as a semi-quantitative control [54].

Documentation

Embryos and frontal adult brain sections were viewed under a Zeiss microscope equipped with Normaski optics (Athens, Greece) using 20×, 40×, and 63× oil immersion objectives. Images were digitized with a CCD camera (Axiophot) (Athens, Greece). Confocal images were captured using a Leica TCS SP2 (Athens, Greece) or a Biorad confocal laser scanning microscope (Athens, Greece). Figures were assembled using Adobe Photoshop.

Results

Directed Expression and Accumulation of Pig and Mouse BM88/CEND1 Transgenes in the Embryonic Nervous System

Pig BM88/CEND1 is a 140-amino-acid protein whereas mouse BM88/CEND1 consists of 149 amino-acids. The two proteins are homologous, showing 64.7% identity and 70.6% similarity, and both possess a transmembrane domain at the C-terminus (Fig. 1A). It has been reported that BM88/CEND1 is weakly expressed in neuronal progenitor cells and becomes prominent in mature post-mitotic neurons. BM88/CEND1 expression is interestingly correlated with the progression of the progenitor cells towards differentiation, indicating that the molecule may function in neurogenesis [36]. Thus, to mimic the native mode of mammalian BM88/CEND1 expression within the *Drosophila* embryo, UAS-BM88/CEND1 transgenic flies were crossed with two neuronal GAL4 drivers, *elav*^{c155}- and *achaete*-GAL4. *elav*^{c155}-GAL4 is a widely used, pan-neuronal driver which is 'leaky', weakly expressing as early as in the neuroectoderm [53] but is strongly expressed in terminally differentiated neurons [55]. *achaete*-GAL4 drives expression in the proneural clusters and later in the *achaete*-expressing Nbs of the embryonic nervous system [53] (see also Figure S1). RT-PCR, western blot analysis, and antibody staining revealed that embryos stably expressed mouse and pig BM88/CEND1 under the control of *elav*^{c155}- and *achaete*-GAL4 drivers from early stages of neurogenesis. Lack of *Drosophila* proteins cross-reacting with either rabbit polyclonal or mouse monoclonal anti-BM88/CEND1 antibodies is consistent with the notion that *Drosophila* lacks a highly conserved homolog (Fig. 1B–C).

Expression of BM88/CEND1 under the control of *elav*^{c155}-GAL4 revealed protein accumulation from embryonic stage 9 onwards with strong expression in mature neurons (Fig. 1D–L). This expression profile is consistent with that previously published by the authors [53]. BM88/CEND1 protein appeared to be cortical (Fig. 1G', I'), suggesting a preferential association with the membrane, presumably due to the presence of the transmembrane domain at the C-terminal ends of both the mouse and pig BM88/CEND1 sequences (Fig. 1A) [35, 39]. Expression of pBM88/CEND1 under the control of *achaete*-GAL4 (Fig. 1M–P) was initially detectable during embryonic stage 9 (Fig. 1M, N) on the neuroectoderm and later in stages 10 and 11 predominantly in the MP2 precursors and MP2 progeny (Fig. 1O, P, S1).

BM88/CEND1 Gene Expression Induces Morphological Defects in the Embryonic CNS

During *Drosophila* embryogenesis, the central and peripheral nervous systems develop from the ventrolateral ectoderm beginning at stage 9 [8, 56]. Accumulation of both mouse and pig BM88/CEND1 driven by the *elav*^{c155}-GAL4 and *achaete*-GAL4 lines resulted in marked embryonic lethality. Staining of these embryos with nervous system-specific markers (anti-HRP, BP104, and anti-Elav) showed extensive but variable neural defects (Table 1, Fig. 2). The anti-HRP marker targets a neuronal membrane protein; the BP104 antibody recognizes the cytoplasmic domain of neuroglia and is an exceptionally useful PNS (peripheral nervous system) marker; and anti-Elav is a marker specific to neuronal nuclei (information obtained from Developmental Studies Hybridoma Bank, DSHB,

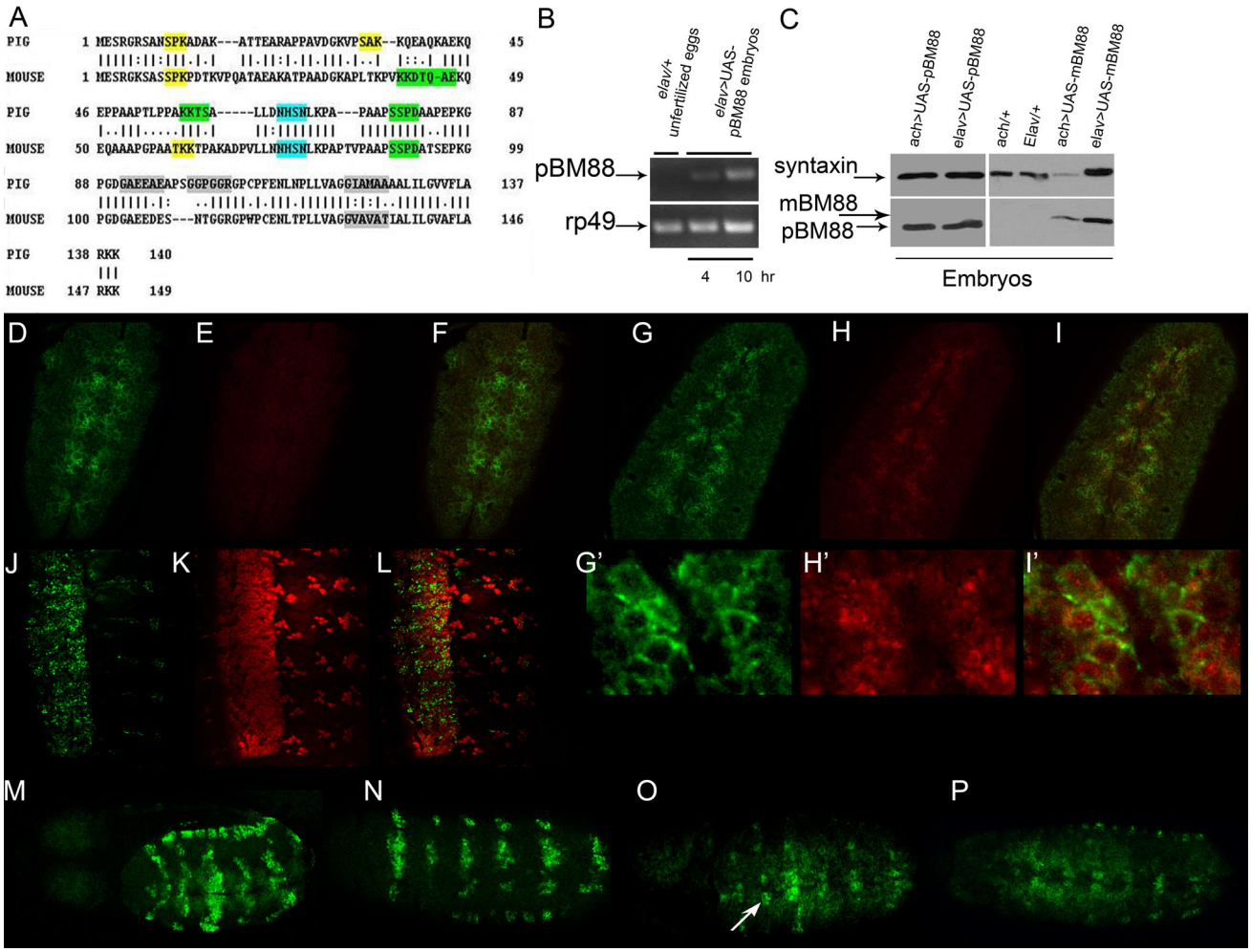


Fig. 1 Expression of mouse and pig BM88/CEND1 in *Drosophila* embryos under the control of *elav*^{c155}- and *achaete*-GAL4 drivers. **A** Comparison of pig and mouse BM88/CEND1 amino-acid sequences. Pairwise sequence alignment was performed using the Emboss program of the European Bioinformatics Institute (EBI, www.ebi.ac.uk/). The two homologous proteins share potential sites: PKC phosphorylation sites (yellow), cAMP- and cGMP-dependent protein kinase and casein kinase II phosphorylation sites (green), N-glycosylation sites (turquoise), and N myristoylation sites (grey). The transmembrane domain spans the region between amino-acids 116 to 136 for pig BM88 and amino-acids 125 to 145 for mouse BM88. **B** RT-PCR analysis of unfertilized eggs and embryos expressing pBM88/CEND1. **C** Western blot analysis using protein extracts from embryos expressing pig and mouse BM88/CEND1. Note the absence of cross-reacting bands in the control lanes. **D–F** Late stage 9 embryos expressing pig BM88/CEND1 under the control of *elav*^{c155}-GAL4 stained with anti-pBM88/CEND1 (D, green) and anti-Elav (E, red) antibodies. Red staining is absent because neurons have not been formed at this stage. F is the merged image. **G–I** Early stage 11 embryos expressing mouse BM88/CEND1 under the control of *elav*^{c155}-GAL4 stained with anti-mBM88/CEND1 (G, green) and anti-Elav (H, red) antibodies. Red staining is observed, representing early-formed neurons at this stage. I is the merged image. **G'–I'** Enlarged views of G–I early stage 11 embryos expressing mouse BM88/CEND1 under the control of *elav*^{c155}-GAL4 stained with anti-mBM88/CEND1 (G') and anti-Elav (H'). I' is the merged image. **J–L** Late stage 15 embryos expressing pig BM88/CEND1 under the control of *elav*^{c155}-GAL4 stained with anti-pBM88/CEND1 (**J**) and anti-Elav (**K**). L is the merged image. **M–P** Embryos expressing BM88/CEND1 under the control of *achaete*-GAL4 driver stained with anti-pBM88/CEND1 (green). **M** Stage 9 embryo expressing BM88/CEND1 throughout the neuroectoderm (dorsal view). **N** Late stage 9 embryo expressing BM88/CEND1 throughout the neuroectoderm (ventral view). **O** Embryo at stage 10 expressing BM88/CEND1 weakly in the neuroectoderm and strongly in the MP2 precursors (arrow) (dorsal view). **P** Embryo at stage 11 expressing BM88/CEND1 in the MP2 precursors. Some weak expression is observed in the neuroectodermal surrounding region between the MP2 precursors (ventral view).

Iowa city, Iowa). Broader neural defects appeared with the *elav*^{c155}-GAL4 than the *achaete*-GAL4 line, whose expression pattern was more confined. BM88/CEND1 accumulation under *elav*^{c155}-GAL4, during embryonic stage 10, led to a premature increase of neural-specific antigenicity of the neuroectoderm, evidenced by elevated staining against the neural-specific marker anti-HRP [57]. Either pig or mouse BM88/CEND1 accumulation led to similar increases in antigenicity (Fig. 2D, G). A similar increase in neural-specific antigenicity of the neuroectoderm was also

Table 1 The phenotypes of BM88/CEND1 expression in *Drosophila* embryos.

Parental genotype	Phenotype			Embryonic lethality* *n* (%)
	+ [%]**	++ [%]**	+++ [%]**	
elav^{C155}-GAL4> UAS-mBM88	Increased neuroectodermal antigenicity at early stages 9/10 [5.6%]	Localized loss of neurons and neuromeric parts at later stages >12 [13.3%]	Severe defects in CNS (VNC and brain lobe) formation at late stages >12 [4.6%]	195 (23.8%)
elav^{C155}-GAL4> UAS-pBM88	Increased neuroectodermal antigenicity at early stages 9/10 [6.1%]	Localized loss of neurons and neuromeric parts at later stages >12 [15.9%]	Severe defects in CNS formation at late stages >12. Prominent phenotype of pBM88 expression [7.0%]	214 (29.1%)
achaete-GAL4> UAS-mBM88	Increased neuroectodermal antigenicity at early stages 9/10 [5.0%]	Localized loss of neurons and neuromeric parts at later stages >12. Defects in formation of connectives and commissures [11.1%]	Severe defects in CNS formation at late stages >12 [3.8%]	180 (20.1%)
achaete-GAL4> UAS-pBM88	Increased neuroectodermal antigenicity at early stages 9/10 [5.4%]	Localized loss of neurons and neuromeric parts at later stages >12. Defects in formation of connectives and commissures [16.0%]	Severe defects in CNS formation at late stages >12. Prominent phenotype of pBM88 expression [5.8%]	223 (26.6%)

+mild phenotype at early embryonic stages.

++moderate phenotype.

+++severe phenotype.

n indicates the total number of embryos observed.

*Data presented for embryonic lethality were obtained using one strong independent line for each UAS transgene.

**Percentage of observed phenotype. Data were obtained using one strong independent line for each UAS transgene, as presented for embryonic lethality.

observed in *achaete*-driven BM88/CEND1-expressing embryos (Fig. 3). Interestingly, an increase in neuroectoderm antigenicity has also been reported for embryos overexpressing the proneural gene *daughterless* [58], suggesting that BM88/CEND1 may interfere with the proneural protein pathway.

In later stage embryos (stage 12 onwards) expressing mouse and pig BM88/CEND1 under *elav*^{c155}-GAL4, CNS defects were apparent (Fig. 2E–L). The defects included a clear decrease in the density of the VNC as shown for embryos in stages 12/13 (Fig. 2F, I, J), 14 (Fig. 2E) and 17 (Fig. 2L) compared to control embryos (Fig. 2B, C, J'). The VNC was frequently fragmented with entire neuromeric parts often missing (Fig. 2E). Severe cases included a complete disorganization of the VNC (Fig. 2H). Brain lobe defects were also striking (Fig. 2F, H, I). Embryos with severe CNS defects also exhibited PNS defects (shown in Fig. 2K, as revealed with BP104 antibody staining), but in this report we have focused largely on the CNS. a*chaete* GAL4-driven mouse and pig BM88/CEND1 expression in embryos yielded defects in the CNS with the connectives of the VNC interrupted in a segmentally repeated pattern (Fig. 2O, O', Q, Q') perhaps due to the strong expression of the *achaete* driver in certain neuronal precursors (Fig. 1) giving rise to apparently incompletely formed, thinner, and often absent commissures (Fig. 2P, P', R, R', S). These results are consistent with those obtained with *elav*^{c155}-GAL4 with the notion that they were precipitated because the number of neurons required to generate the normal VNC was reduced.

Overall, differential phenotypes were observed with mouse or pig BM88/CEND1 accumulation in *Drosophila* embryos. This is highly significant since the two BM88/CEND1 proteins differ in primary sequence [38] and this is the first time that they have been shown shown to be functionally homologous in the context of a living organism. The phenotypic variability between mouse and pig BM88/CEND1 is unlikely to be attributable to different accumulation levels of the two proteins as shown in representative figure (Fig. 1A), and may be a consequence of differential functional efficiency arising from the differences in their primary sequence (Fig. 1A) [38]. The CNS defects varied from mild- to- severe in different embryos with the porcine protein consistently producing stronger phenotypic effects (Fig. 2). All morphological defects as a result of BM88/CEND1 accumulation occurred at random locations in the CNS and were restricted to the nervous tissue. It is unlikely that the defects were due to

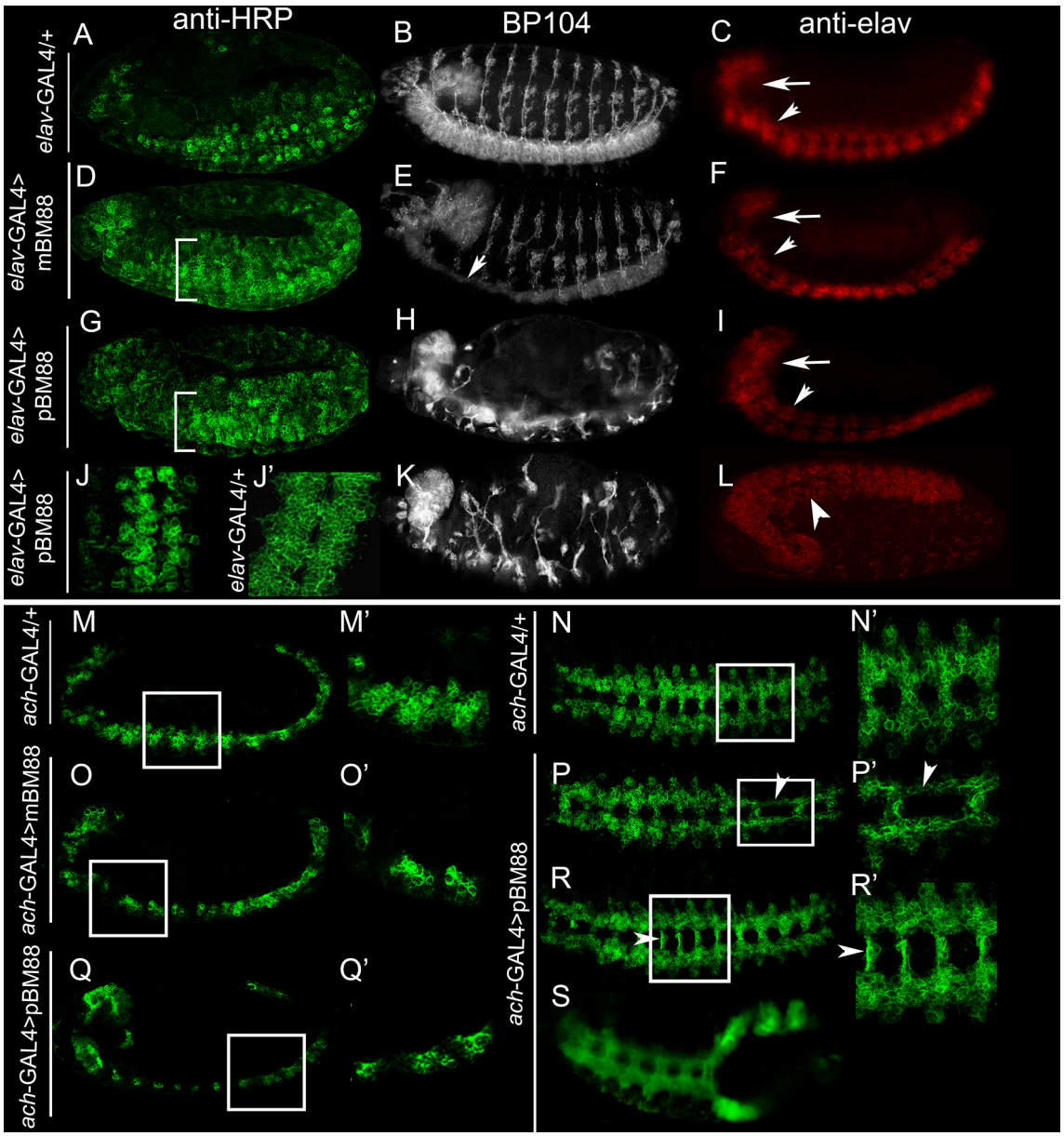
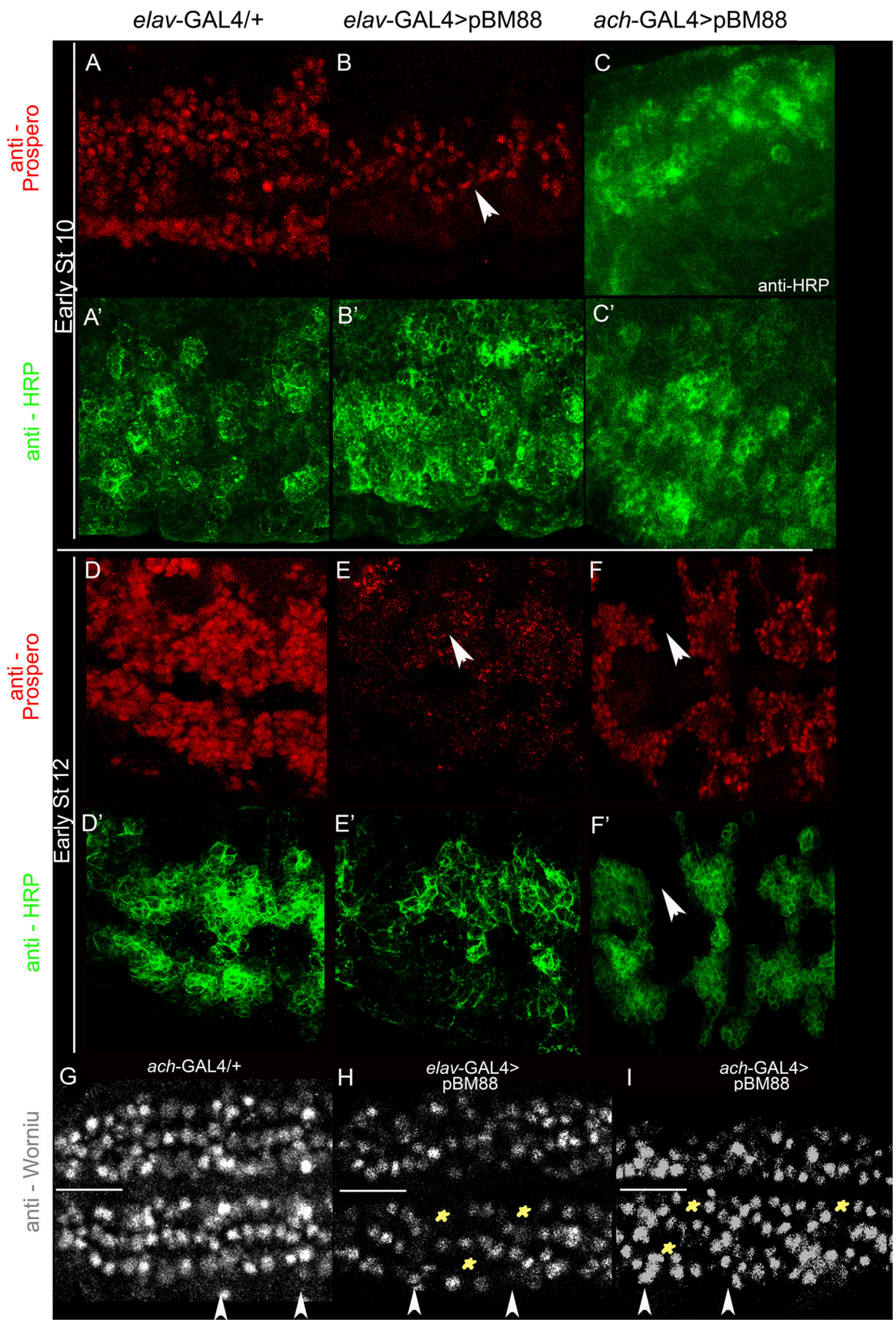


Fig. 2 Morphological defects of the embryonic CNS induced by accumulation of mammalian BM88/CEND1. The CNS morphology of all embryos was visualized using the neuronal markers anti-HRP (green), BP104 (grey), and anti-Elav (red). **A–I**, **K**, **M**, **M'**, **O**, **O'**, **Q**, and **Q'**, lateral views; **J**, **J'**, **N**, **N'**, **P**, **P'**, **R**, **R'**, and **S**, ventral views; **L**, ventro-lateral view. Anterior is to the left. Defects in brain morphology are indicated by arrows. Localized loss of neurons and neuromeric parts are indicated by arrowheads. n = 8 observed embryos at stage 10, n = 15 embryos at stages 12–17. **A–C** Wild-type embryos at stages 10, 14/15, and 12/13. **D–L** Embryos expressing BM88/CEND1 under the control of *elav*^{c155}-GAL4. **N–S** Embryos expressing BM88/CEND1 under *achaete*-GAL4. **D**, **G** Embryos (stage 10) expressing mouse and pig BM88/CEND1. Note the enhanced HRP fluorescence indicating an increase of neural-specific neuroectoderm antigenicity (square brackets), in comparison to controls in **A**. **E**, **F** Embryos at stages 14/15 and 12/13 expressing mBM88/CEND1. Note the defects in VNC formation. **H**, **I** Embryos at stages >14/15 and 12/13 expressing pBM88/CEND1. Severe defects in the VNC and brain lobes are present. The embryonic stage in **H** was difficult to determine due to phenotypic severity. **J**, **J'** VNCs of embryos (stage 12) expressing pig BM88/CEND1 and wild-type controls. **K** Embryo at stage >14/15 expressing pBM88/CEND1 (embryonic stage difficult to determine due to phenotypic severity). Defects in the CNS and PNS are presented. **L** Embryo at stage 17 expressing pBM88/CEND1 under the control of *elav*^{c155}-GAL4. Note the localized loss of neurons. **O**, **Q** Embryos expressing mouse and pig BM88/CEND1 under *achaete*-GAL4 driver. **O'**, **Q'** Enlarged views of selected parts of **O** and **Q**. Note the segmental reduction in neurons following expression of *achaete*-GAL4. **P**, **R** Ventral views of embryos expressing pBM88/CEND1. The connectives and commissures appear to be thinner than the control (**N**, **N'**) as also indicated by the squares (in **P**, **R**) and by the arrowheads (**P'**, **R'**). **P'**, **R'** Enlarged views of selected parts of **P** and **R**. **S** A more severe phenotype is presented upon pBM88/CEND1 expression where the two main connective tracts are fragmented longitudinally and disconnected.


elav-GAL4/+
elav-GAL4>pBM88
ach-GAL4>pBM88
anti - Prospero
anti - HRP
Early St 10
A
B
C
anti-HRP
A'
B'
C'
anti - Prospero
anti - HRP
Early St 12
D
E
F
D'
E'
F'
anti - Worniu
G
ach-GAL4/+
H
elav-GAL4> pBM88
I
ach-GAL4> pBM88

◀**Fig. 3** Effect of BM88/CEND1 expression on precursor cell formation. Control and BM88/CEND1-expressing embryos were doubly labeled with anti-Prospero (red) and anti-HRP (green). Anterior is to the left. The genotypes of the embryos are shown on the top and all embryos expressed pig BM88/CEND1 (n = 13 for each genotype and stage presented). **A**, **A'**, **B**, **B'** Early stage 10 embryos expressing pBM88 under the *elav* ^{c155}-GAL4 driver (**B**, **B'**) and exhibiting increased antigenicity of the neuroectoderm in comparison with controls (**A**, **A'**), occurring at the expense of the precursor cells since their number was significantly reduced. **C**, **C'** Early stage 10 embryos expressing pBM88 *achaete*-GAL4 driver, lateral and ventral views. **E**, **E'** Early stage 12 embryos expressing pBM88/CEND1 under *elav*^{c155}-GAL4. Note the marked reduction of prospero staining compared to control embryos (**D**, **D'**). **F**, **F'** Similarly staged embryos expressing pBM88/CEND1 under *achaete*-GAL4. A dramatic reduction of precursor cells (arrowheads in **E** and **F**) as well as of neurons (arrowheads in **E'** and **F'**) is apparent in the embryos expressing BM88/CEND1. **G–I** Worniu staining of stage 11 wild-type embryos (**G**), pBM88/CEND1-expressing embryos under *elav*^{c155}-GAL4 driver (**H**) and under *achaete*-GAL4 driver (**I**) to visualize neuroblasts (n = 12 each). The pBM88/CEND1-expressing embryos under the control of either GAL4 driver display defective formation of neuroblasts. Asterisks in yellow (**H**, **I**) represent areas where neuroblasts are missing. The line shows the midline region. Arrowheads indicate neuromere hemisegments.

general toxicity precipitated by BM88/CEND1, since accumulation of either protein in the mesoderm and muscles using *how24B*- and *mef2*-GAL4 drivers resulted in fully viable embryos without evident morphological defects in either the mesoderm or somatic musculature or the midgut or even the CNS.

In conclusion, our data suggest that expression of the neuronal-specific BM88/CEND1 molecule is sufficient to specifically interfere with normal development of the embryonic CNS, indicating that BM88/CEND1-interacting proteins likely exist in the *Drosophila* embryonic nervous system and mediate such defects.

BM88/CEND1 Transgene Expression Affects Neuronal Precursor Cell Formation

To determine whether the apparent drastic loss of neurons in BM88/CEND1-expressing embryos was solely due to defective precursor cell differentiation, we doubly labeled the embryos with the neuronal anti-HRP marker and anti-Prospero, a general marker for neuronal precursor cells, parental Nbs, and GMCs. Early stage 10 pBM88/CEND1-expressing embryos under *elav*^{c155}-GAL4, showed a marked decrease in the Prospero signal (Fig. 3B) suggesting defective formation of GMC/Nbs. However, this was accompanied by increased antigenicity of the neuroectoderm indicated by anti-HRP staining (Fig. 3B'). Increased neuroectodermal antigenicity was also observed in pBM88/CEND1-expressing embryos under the *achaete*-GAL4 driver (Fig. 3C, C'). Reduction in anti-Prospero staining also became evident in early stage 12 embryos (Fig. 3E, F) and was accompanied by a reduction in the neuronal specific anti-HRP signal (Fig. 3E', F'). Congruently, the fragmented VNC exhibited by stage 12 *achaete*-GAL4>BM88/CEND1-expressing embryos was characterized by an overall reduction of anti-Prospero staining around the region where neurons had failed to form (Fig. 3E, E'). These data indicate that the loss of neurons in *Drosophila* embryos resulted from failure to form GMCs or parental Nbs or a combination of the two.

To discriminate among these possibilities, we used an antibody against Worniu protein which is expressed in all Nbs [52, 59–61], thus assessing Nb formation in BM88/CEND1-expressing embryos (Fig. 3H, I). We focused on embryos expressing BM88/CEND1 using the broad neuronal *elav*^{c155}-GAL4 driver but also the *achaete*-GAL4 line because, first, we wanted to determine whether the effects of BM88/CEND1 were restricted to the cells where this protein accumulates, and second, whether these effects could be easily assessed in a specific context such as the well-defined MP2 precursors [62]. Targeted expression of BM88/CEND1 under the *elav*^{c155}-GAL4 driver revealed defective formation of Nbs in stage 11 embryos (Fig. 3H). Formation of lateral Nbs but also around the midline was clearly impaired. Moreover, expression of BM88/CEND1 under *achaete*-GAL4 also resulted in slight defects in the formation of Nbs as stained with anti-Worniu (Fig. 3I). Counting of Nbs per hemisegment revealed the presence of 23 Nbs–25 Nbs in all control embryos analyzed (Fig. 3G). *elav*^{c155}-GAL4>pBM88-expressing embryos showed an average of 14 Nbs–15 Nbs per hemisegment whereas in *achaete*-GAL4>pBM88-expressing embryos, an average of 19 Nbs–20 Nbs per hemisegment were counted. These differences between the two GAL4 drivers at embryonic stage 11 (when all Nbs are finally formed) may be related to the broader and stronger expression profile of the *elav*^{c155}-GAL4 line in the region compared to that of the *achaete*-GAL4 line (see also Fig. 1). However, in the majority of embryos, the reduction of anti-Worniu staining was never as severe as the reduction of the Prospero signal (Fig. 3B, E, F). These results suggested that although Nb formation was likely affected by BM88/CEND1 expression, the formation of GMCs was more severely impaired than Nb formation, further suggesting that BM88/CEND1 accumulation affects the formation of neuronal precursor cells, but mostly GMCs.

BM88/CEND1 Transgene Expression Affects MP2 Precursor Formation

To resolve the effects of BM88/CEND1 accumulation during the formation and differentiation of Nbs and GMCs with more precision, we specifically focused on the MP2

precursors. These cells are specified at early stage 9, but then atypically, they undergo a single division at late stage 11, generating two intersegmental interneurons (dMP2 and vMP2) that bifurcate and send axons in the anterior and posterior directions and by stage 17, span the entire length of CNS [62]. The *achaete*-GAL4 driver was well-suited to focus on the fate of these cells. After Nb delamination, 4 Nbs per hemisegment, the MP2 precursor, and 3 Nbs–5 Nbs, 7 Nbs–1 Nbs, and 7 Nbs–4 Nbs retained *achaete* expression. In addition, *achaete* was maintained by MP2 and by 3 Nb–5 Nb until stage 10 [63, 64]. Transgene expression driven by *achaete*-GAL4, appeared to mirror the native *achaete* expression pattern [53], but it also persisted in differentiated MP2 cells (vMP2 and dMP2) until formation of the longitudinal axonal tracts from the dMP2 interneurons in later stages of embryogenesis (13–15) (Fig. S1).

To probe the fate of the MP2s in embryos expressing BM88/CEND1 under *achaete*-GAL4, we tracked the Nb-specific marker Worniu in these cells. Cells expressing BM88/CEND1 under *achaete*-GAL4 were identified either by accumulation of the BM88/CEND1 protein itself, or the reporter protein bovine Tau (bTau) which was used to monitor MP2 formation and differentiation in control embryos. Neurons were identified by anti-HRP staining (Fig. 4, S1). In late stage 10 embryos, both bTau and BM88/CEND1 were expressed weakly in the neuroectoderm, but as expected, strongly in the MP2 precursors (Fig. 4A, C). Furthermore, cells in the expected locale of MP2 precursors expressed BM88/CEND1 under *achaete*-GAL4, but lacked Worniu staining (Fig. 4C', C''), in stark contrast to control embryos (Fig. 4B). The cortical appearance of BM88/CEND1 in MP2s was noticeable (Fig. 4C, C'), likely due to BM88 binding to the cell membrane and in agreement with the observations in Fig. 1. In contrast, bTau was cytoplasmic within MP2s as expected (Fig. 4A, A', A'').

In addition, in a large number of similarly staged embryos, the MP2s (or MP2-like cells) had prematurely migrated towards the midline where they were abnormally fused (Fig. 4C). This abnormal cluster accumulated BM88/CEND1 protein (Fig. 4C, white arrow) as well as neural-specific markers such as HRP (Fig. 4D, white arrow). By late stage 11, pBM88/CEND1-accumulating MP2 precursors exhibited defective differentiation because they appeared irregular (Fig. 4E) and distinctly different from similarly staged control embryos accumulating bTau (Fig. 4F), where MP2s differentiate normally having properly migrated towards the midline at stage 11. Most likely then, the defective MP2-like differentiation in stage 11 embryos was a result of defectiveNb formation illustrated by loss of Worniu or else due to early/premature differentiation of these cells obtaining neural (or neural-like) characteristics as revealed by anti-HRP staining (Fig. 4D).

Taken together, these results indicated that BM88/CEND1 apparently engages conserved molecular constituents and disrupts signaling cascades important for the initial formation and subsequent differentiation of MP2 precursors. Similar phenotypic defects have been previously described in mutant embryos with defective *achaete/scute* protein complex function [65] or defective EGF receptor signaling [62].

BM88/CEND1 Transgene Expression Interferes with Cell-Cycle Regulation of Precursor Cells

As proposed for vertebrate model systems [42], BM88/CEND1 accumulation in early embryos could interfere with the cell cycle of precursor cells leading to the observed defects in Nb and subsequent GMC formation. To address this possibility, the pattern of mitotic cells of the neuroectoderm was investigated using cyclin A expression [66] and phosphohistone 3 (PH3) as markers for cell-cycle progression. In stage 11 control embryos, cyclin A was distributed throughout the neuroectoderm (Fig. 5A') which encompassed the neuroectodermal precursor cells and delaminated Nbs. At that stage, young post-mitotic neurons forming part of the VNC had already appeared and stained positive with anti-HRP (Fig. 5A). In embryos expressing pBM88/CEND1 at stage 11 (Fig. 5B'), the segmental expression pattern of cyclin A appeared highly disorganized. Congruence with this, the segmented pattern of PH-3 positive mitotic cells in stage 11 control embryos (Fig. 5C) was significantly disrupted and visibly reduced in embryos expressing pBM88/CEND1 (Fig. 5D). Later at stage 14 when the CNS differentiates (Fig. 5E, F), cyclin A was expressed at low levels in small clusters of remaining actively-dividing Nbs in the lateral regions of the nerve cord (Fig. 5E'). In embryos expressing pBM88/CEND1 at stage 14 (Fig. 5F'), the expression pattern of cyclin A appeared aberrantly clustered and highly disorganized.

The above results indicate that accumulation of BM88/CEND1 in the neuroectoderm can induce defects in cell-cycle progression and/or the regulation of the neuronal precursor cells, possibly leading to a state of premature exit and subsequent differentiation, as evidenced by a general increase in neuroectodermal antigenicity (Figs. 2 and 3). This could then likely be the reason for the defective formation of precursor cells, including the MP2-like precursors (Figs. 2–4).

BM88/CEND1 Inhibits MAPK Activation During *Drosophila* Embryonic Neurogenesis

During early embryogenesis, MAPK receives signals from the EGF receptor acting *via* the conserved RAS/RAF pathway, thus regulating a number of developmental

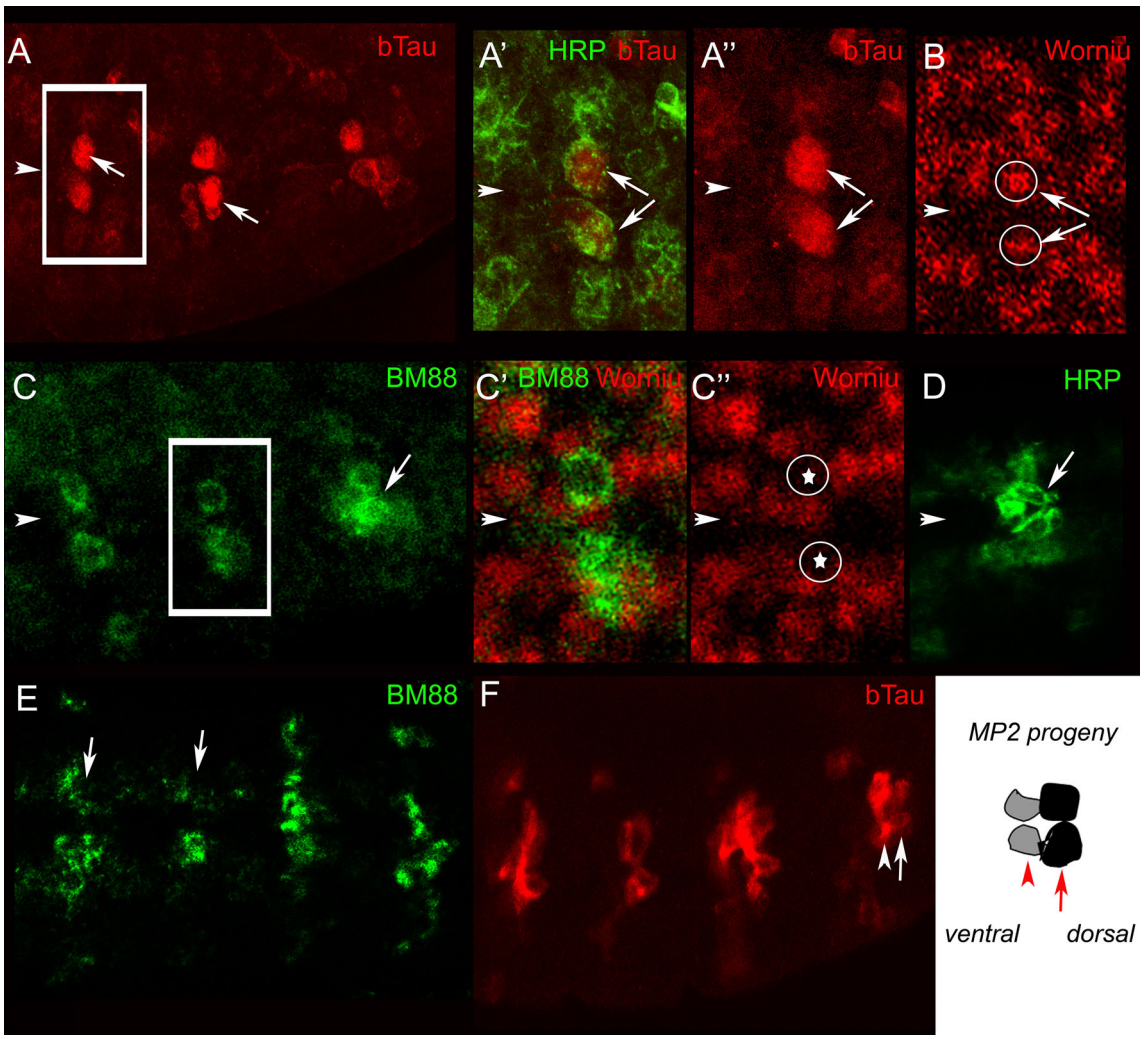


Fig. 4 Effect of BM88/CEND1 accumulation on MP2 precursor formation. Anterior is to the left. **A**, **A'**, **A''** *achaete*-GAL4 expression in control embryos by double labeling with anti-bTAU (red) and anti-HRP (green), unless otherwise stated. At stage 10, MP2 precursors were clearly specified in each hemisegment as reported by bTAU expression (arrows) without any fusion defects (n = 15). **A', A''** Enlarged views of the boxed area in **A**. **B** Worniu staining of MP2 precursors (indicated by circles) in a control *achaete*-GAL4>bTAU embryo (n = 15). **C**, **C'**, **C''** *achaete*-GAL4>pBM88/CEND1 embryos at stage 10 doubly labeled with anti-BM88/CEND1 (green) and anti-Worniu (red). **C** MP2 precursors (in a single segment) expressing pBM88/CEND1 were aberrantly in close proximity to each other and abnormally fused (arrow). The rightward arrowheads show the midline (n = 12). **C'** and **C''** are enlarged views of the boxed area in **C**. The MP2-like precursors expressed pBM88/CEND1 but Worniu staining was completely absent (indicated by the circled 'stars' in **C**''), in contrast to control MP2s which expressed Worniu (**B**). **D** MP2-fused phenotype of an *achaete*-GAL4>pBM88/CEND1 embryo (n = 12 embryos). Such a cell cluster can express the neural-specific marker anti-HRP (arrow). **F–G** *achaete*-GAL4>pBM88/CEND1 and *achaete*-GAL4>bTAU embryos during stage 11 stained for BM88/CEND1 and bTAU (n >15 stage 11 embryos). In the embryo expressing pBM88/CEND1, possibly due to early/premature signaling events, proper MP2 differentiation appears to be defective since MP2s or MP2-like precursors had not migrated properly towards each other expressing BM88/CEND1 at lower levels (arrows in **E**) and in an irregular pattern, in contrast to the *achaete*-GAL4>bTAU embryo which shows normal MP2 differentiation events with the MP2s having properly migrated towards the midline at stage 11 and differentiated. Ventral and dorsal MP2 progeny are indicated by the arrowhead and arrow, respectively (**F**). MP2 progeny are also represented in the scheme.

processes which include patterning of the embryonic neuroectoderm, neuroblast fate specification, and normal CNS development. MAPK is activated by dual phosphorylation of threonine and tyrosine residues by MEK [28, 32, 67, 68]. The MAPK pathway is also known to be active during neuronal differentiation processes *via* regulating certain transcription factors within the nucleus and altering gene expression [26, 68]. There is a broad phenotypic similarity between embryos accumulating BM88/CEND1 and those with impaired EGF receptor signaling [62]. Therefore, we investigated whether the effects of BM88/CEND1 accumulation are precipitated by interference with the EGF receptor signaling pathway. To that end, we assayed for the presence of the activated,

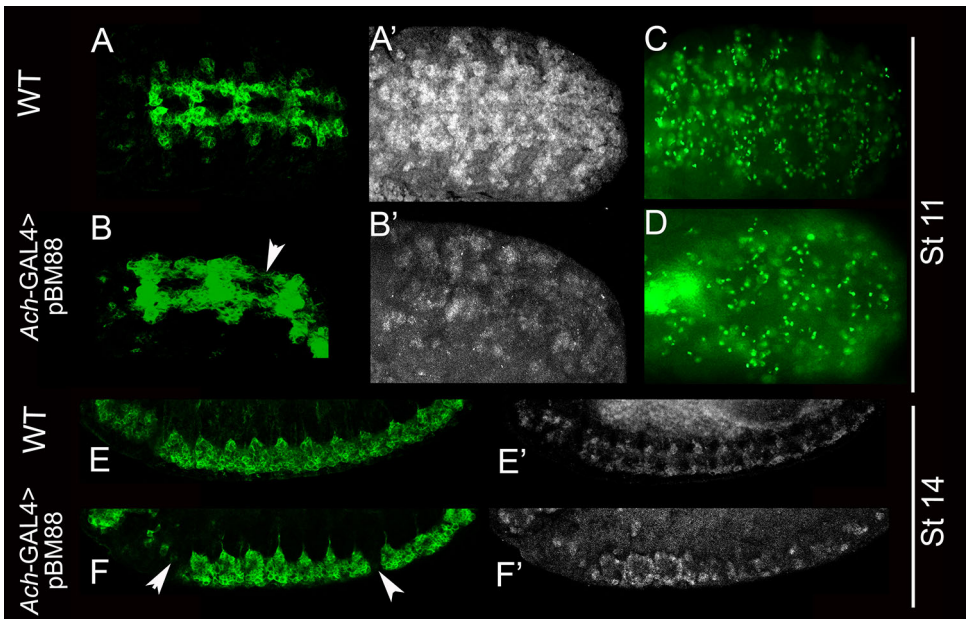


Fig. 5 BM88/CEND1-expressing embryos fail to proceed through the cell-cycle. **A, A'**, **B**, **B'**, **E**, **E'**, **F**, **F'** Embryos doubly stained with anti-HRP (green) to visualize differentiated CNS and morphological defects (arrowheads in **B** and **F**) induced upon BM88/CEND1 expression, as well as with anti-cyclin A (grey) to track the distribution of the cell-cycle regulator cyclin A. Cyclin A-expressing precursor cells at stages 11 (A') and 14 (E') were disorganized in pBM88/CEND1-expressing embryos (**B'** and **F'**). **C**–**D** Expression of the mitotic marker phosphohistone H3 at stage 11. Mitotic cells were present in a segmentally-repeated pattern in control embryos (**C**). In pBM88/CEND1-expressing embryos, mitotic cells appeared to be reduced and disorganized (**D**). Anterior is to the left. $n = 12$ for each genotype analyzed and for each embryonic stage presented.

diphosphorylated form of MAPK during neurogenesis [62, 69]. In control embryos, during the initial stages of neurogenesis (early embryonic stage 9), activated MAPK appeared mostly in ventral neuroectodermal cells (Fig. 6A, A', C, C'). At stage 10, di-phosphorylated MAPK was still present in the neuroectoderm, predominantly in the most medial neuroectodermal cells that surround the midline but also in the tracheal placodes (Fig. 6E, E'). In contrast, di-phosphorylated-MAPK staining was dramatically reduced in embryos expressing BM88/CEND1 (Fig. 6B, B', D, D', F, F', G). These results indicated that accumulation of BM88/CEND1 impedes MAPK phosphorylation and thus activation, possibly by interfering with EGFR/MAPK signaling. This interpretation is consistent with and can explain the BM88/CEND1-dependent interference with the formation of neuronal precursors and CNS formation by inducing defects in cell-cycle progression and/or regulation of the neuronal precursor cells (Fig. 5).

BM88/CEND1 Accumulation Disrupts Eye Disc Morphology

To investigate the action of mammalian BM88/CEND1 function on post-embryonic developing cells in *Drosophila*, we used the eye discs that provide a different developmental context. *Drosophila* eye discs are an appropriate model to observe functional effects since their morphological alteration may lead to viable and readily visible phenotypes [15, 25, 70]. Targeted expression of mouse and pig BM88/CEND1 in the eye disc was achieved by using *GMR*-GAL4 fly lines. The GMR promoter sequences are able to drive transgene expression throughout the entire eye disc. Thus, *GMR*-GAL4-driven expression of mouse and pig BM88/CEND1 induced a weak 'rough eye' phenotype (Fig. 7A–C). To further analyze this eye defect, we examined the morphology of the inner structure by hematoxylin-eosin staining of horizontal sections of adult eyes (Fig. 7D, E). The Optic lobe morphology of eyes expressing BM88/CEND1 was substantially affected. The retina appeared to be disorganized and the photoreceptor rhabdomere structure was frequently disrupted. The overall size of the lamina was reduced compared to the wild type. The pigment cell feet comprising the fenestrated membrane (to which the photoreceptors and the cone cells are attached) and the layer of pigmented subretinal cells found below the basement membrane were greatly separated (Fig. 7D, D', E, E') [71, 72]. Both layers of the pigmented cell feet and the subretinal cells seemed to be lost or severely malformed. Furthermore, retinal photoreceptors were missing from the lamina region below the fenestrated basement membrane as visualized by anti-Elav staining of horizontal sections of adult eyes, revealing reduced numbers of lamina neuronal nuclei in that region (Fig. 7G, G'). Interestingly, optic lobe morphology

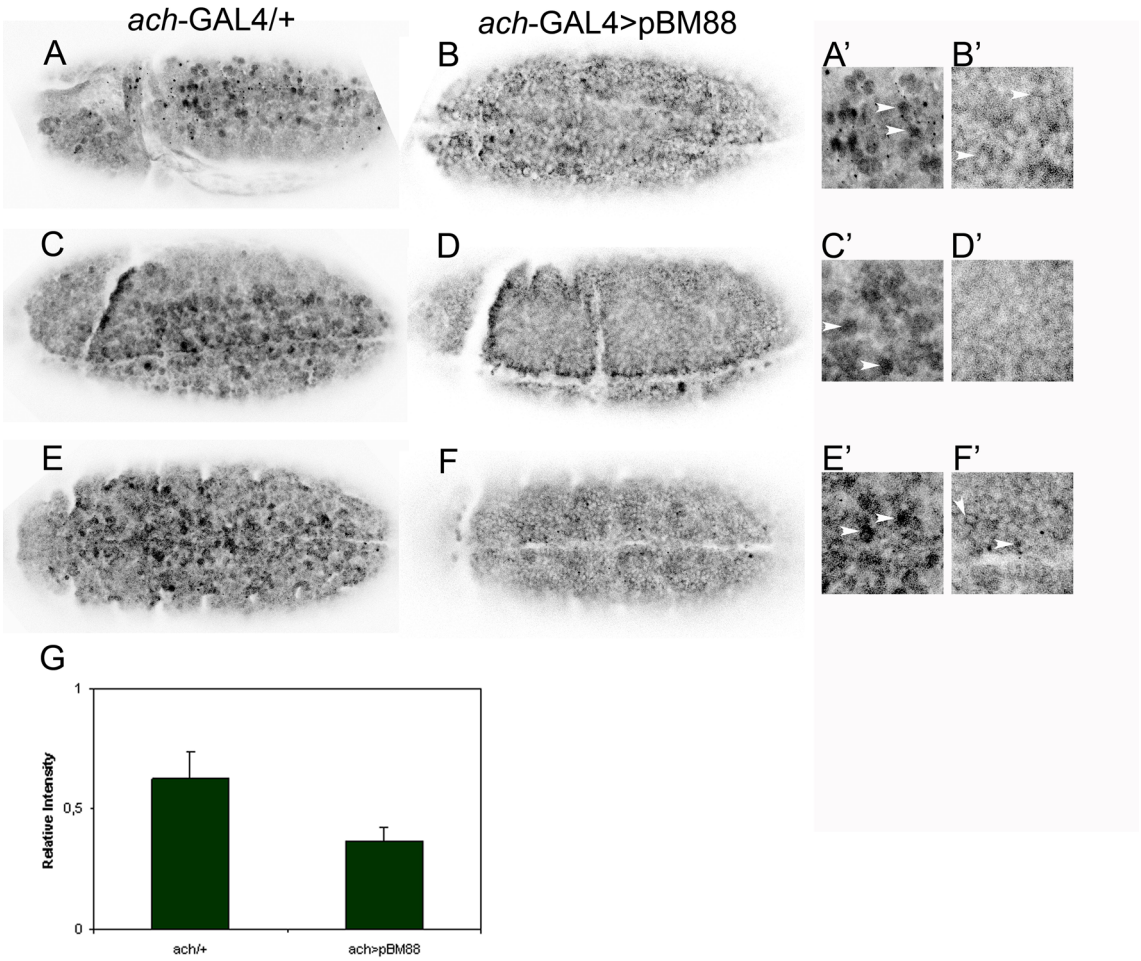


Fig. 6 Expression of pBM88/CEND1 abolishes MAPK activation in the ventral neuroectoderm during initial embryonic neurogenesis. **A**, **C**, **E** Views of control embryos stained for di-phosphorylated-MAPK. **A'**, **C'**, **E'** Enlarged views of selected parts of **A**, **C**, and **E**. **B**, **D**, **F** Embryos expressing pBM88/CEND1 under the *achaete*-GAL4 line. Active MAPK staining was dramatically reduced. **B'**, **D'**, **F'** Enlarged views of parts of **B**, **D**, and **F**. **G** Relative quantification of active MAPK staining in control and pBM88/CEND1-expressing embryos by the *achaete*-GAL4 line ($n = 5$, $P < 0.01$).

appeared to be normal when BM88/CEND1 was expressed using the *sevenless*-GAL4 driver (Fig. 7C''). This phenotypic difference was likely due to variations in the strength and site of transgene expression defined by the GMR and *sevenless*-GAL4 drivers. The former is expressed in the adult eye disc and in the third-instar imaginal disc (from behind the morphogenetic furrow) in all actively-dividing cells as well as terminally-differentiated cells, whereas the latter is expressed predominantly in post-mitotic photoreceptor and cone cells [73], further indicating that the functional effect BM88/CEND1 expression may be on precursor cells, although more recent studies have shown that these commonly used eye-specific *GMR*-GAL4 and *sevenless*-GAL4 drivers are also expressed in tissues other than eyes [74].

The above results showed that BM88/CEND1 protein expression in the optic lobe can induce disorganized morphology of the retinal pattern characterized by disrupted photoreceptor axons and malformed accessory pigment cells. Although the developmental onset and the exact causative process of these observed abnormalities (e.g. defective cell cycle and/or differentiation) have not been addressed yet, our data, in agreement with the data using the embryonic CNS, suggest that BM88/CEND1 can interfere with the normal development of various cell types arising from neuronal precursor cells in the developing *Drosophila* eye. This may be achieved by affecting precursor cells involved in universal signaling cascades such as that of the EGFR-activated [28–32] and/or the proneural/Notch signaling pathway associated with cell fate, differentiation, and developmental processes [43, 75–77].

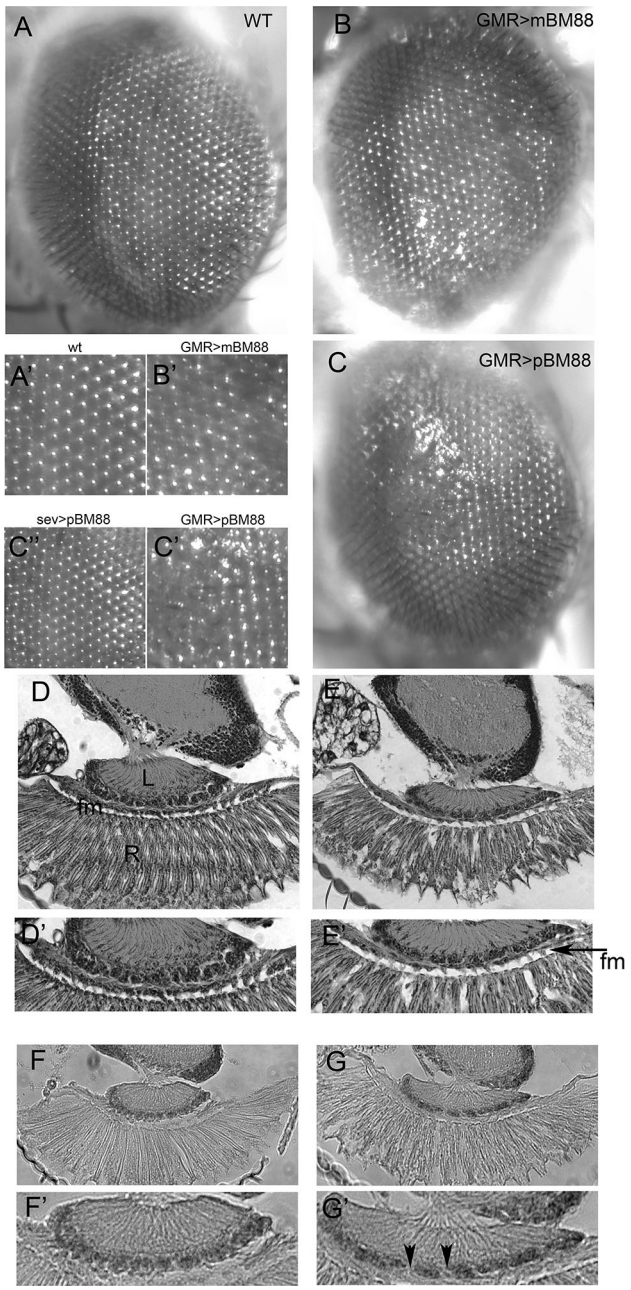


Fig. 7 Targeted expression of mammalian BM88-induced disruption of normal eye disc morphology. **A–C** GMR/+ (WT), GMR>mBM88, and GMR>pBM88 expression of mouse and pig BM88 in the eye disc using the GMR-GAL4 driver. **A'–C'** Enlarged views of **A–C**. **C''** Sev>pBM88, expression of pig BM88/CEND1 in the eye disc using *sevenless*-GAL4 driver. **D**, **D'**, **E**, **E'** Horizontal *Drosophila* head sections stained with hematoxylin-eosin. Retinal pattern disorganization was evident in the eyes expressing BM88/CEND1. The size of the lamina was reduced (**E**) and the fenestrated membrane was disrupted in BM88-expressing eyes. D' and E' are higher magnification views of **D** and **E**, clearly showing the collapsed fenestrated basement membrane (arrow). **F**, **F'**, **G**, **G'** Horizontal *Drosophila* sections stained with the neuronal marker anti-Elav. Note the reduction of Elav-positive photoreceptor cells in the lamina region of the optic ganglion expressing BM88 (arrowheads in **G'**). $n = 10$ eyes of each genotype. R, retina; L, lamina; fm, fenestrated basement membrane.

Discussion

BM88/CEND1 appears to be a vertebrate-specific neuronal protein [35, 39] since a *Drosophila* BM88/CEND1 homologue remains to be identified. Our results lead us to hypothesize that a functional homologue actually exists in *Drosophila* and is involved in nervous system development, and that mammalian BM88/CEND1 mimics its effects. Alternatively, there is no *Drosophila* BM88/CEND1 homologue, but expression of the mammalian BM88/CEND1 can interfere with evolutionarily conserved molecular components of signaling pathway(s) involved in neurodevelopmental pathways. Nevertheless, we have shown that mammalian BM88/CEND1 is functional *in vivo* during *Drosophila* neurogenesis in the embryo. We have excluded the possibility that the BM88/CEND1-dependent deficits in embryonic development are a consequence of non-specific toxicity, because targeted accumulation of either mouse or pig BM88/CEND1 in other embryonic tissues did not cause any morphological defects or lethality. Furthermore, the effect on neuronal precursors seems to be specific, as targeted expression of BM88/CEND1 in late pupal and adult mushroom bodies does not cause neuro-anatomical defects [51] (see also Figure S2). BM88/CEND1, though, had functional effects in *Drosophila* eye, a different post-embryonic developmental context comprising of distinctive cell differentiating fates from neuronal lineages, further suggesting that the effect of BM88/CEND1 may be on neuronal precursor cells. Both mouse and pig BM88/CEND1, despite their primary sequence differences, appear to specifically engage conserved endogenous *Drosophila* cellular components essential for proper formation and differentiation of neuronal precursor cells.

Collectively, our experimental results suggest that BM88/CEND1 accumulation leads to misregulation of the cell cycle in neuronal precursor cells and likely this is achieved, at least in part, by inhibiting MAPK phosphorylation and activation. This then leads to the premature increase in neural-specific antigenicity of neuroectoderm, likely associated with a premature differentiated state, an overall reduction of parental Nbs, and a more pronounced inhibition of GMC formation followed by multiple CNS defects. An alternative interpretation is that first the precursor cells prematurely exit the cell cycle and then these cells no longer respond to EGFR/MAPK signaling. This interpretation would imply that premature differentiation renders the EGFR/MAPK pathway unresponsive/inactive and also that premature differentiation may occur *via* a mechanism which is independent of EGFR/MAPK signaling. Studies, however, have shown that downregulation of MAPK signaling is linked to premature

differentiation [78]. EGFR/MAPK activity is critically required to coordinate proper cell-cycle progression and subsequent correct fate determination and proper patterning of the CNS [23, 24, 28, 79]. In our study, defective precursor cell formation appeared to be associated with defects in cell-cycle progression because programmed cell death did not appear to contribute (Figure S3). These results therefore are consistent with previous studies in cell lines and in chick embryos demonstrating a strong requirement for BM88/CEND1 in neuron-generating processes [37, 42].

Our findings demonstrate that *Drosophila* can be used as a model to investigate the mode of action, processes, and signaling pathways engaged by BM88/CEND1 in a whole-animal model. In fact, our findings that BM88/CEND1 abolished MAPK activation and affected the expression of cyclin A during embryonic neurogenesis supports the notion that BM88/CEND1 may act, at least in part, by interfering with the *Drosophila* EGFR→→MAPK→cyclin D→→cyclin A signaling pathway. This pathway has multiple and essential developmental roles, directing cell fate choices by coordinating cell-cycle progression [28, 79], cell division, cell survival, and migration [26, 28, 30–32]. Moreover, in the epidermis, lowering the levels of active MAPK leads to premature differentiation and proliferative failure [78, 80]. These results are in agreement with early studies indicating altered responsiveness to growth factors after transfection of mouse neuroblastoma cells with BM88/CEND1 [81]. It is, thus, plausible that BM88/CEND1 interferes with EGFR/MAPK signaling since it appears functionally to negatively regulate the pathway. By virtue of its transmembrane domain, which seems to be functional in *Drosophila* embryos, BM88/CEND1 may engage and negatively regulate the EGF-receptor itself directly or indirectly *via* intermediate receptor binding molecules.

Moreover, considering that neuronal (including MP2) precursor specification in *Drosophila* is regulated by another highly conserved pathway, the Notch/proneural (*achaete/scute* complex) signaling pathway [62, 65], the possibility that BM88/CEND1 accumulation interferes with both pathways is also raised. Thus, an alternative but mutually non-exclusive scenario to BM88/CEND1 regulation of EGFR signaling is that BM88/CEND1 is able to interact directly or indirectly with one or more of the proneural proteins which promote the formation of precursor cells during neurogenesis. Such a scenario has been proposed by Politis *et al*. suggesting that BM88/CEND1 functions in the neuronal differentiation of chick embryos acting downstream of the proneural protein pathway [37]. However, as genetic crosstalk between the Notch/proneural and EGFR signaling pathways is known [67], it is also possible that BM88/CEND1 may engage both pathways. In fact, activation of the EGFR pathway in *single-minded* mutant embryos by overexpressing the activating ligand spitz, restores normal *achaete* expression and corrects MP2 precursor specification [62].

More recent experiments by Tsioras *et al*. have shown that functional interactions between BM88/CEND1, RanBPM, and Dyrk1B affect cyclin D1 levels and the balance between cellular proliferation and differentiation in Neuro 2a cells indicating that a similar mechanism may influence cell-cycle progression/exit and the differentiation of neuronal precursors [44]. Future studies will focus on demonstrating a direct association of BM88/CEND1 with the molecular constituents of associated pathways since, to date, the exact molecular action and direct binding partners of BM88/CEND1 have not been identified and characterized and it seems that *Drosophila* may play a crucial role in this identification process. Finally, the fact that the mammalian BM88/CEND1 proteins are active in *Drosophila* neurogenesis indicates the existence of functional interactors in *Drosophila* which participate as determinants in neurogenic process and validates the use of this model system as a powerful tool to study function not only of genes and gene products highly conserved between flies and mammals but also of those not appearing to have counterparts in the fly.

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Conflict of interest The authors declare that they have no conflict of interest.

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