Received: 27 October 2019 | Revised: 23 October 2020 | Accepted: 6 December 2020

DOI: 10.1111/ede.12364

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RESEARCH

A *cis*-regulatory change underlying the motor neuron-specific loss of *Ebf* expression in immotile tunicate larvae

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Funding information

European Molecular Biology Organization, Grant/Award Number: ALTF 1608-2014; Boehringer Ingelheim Fonds; France BioImaging infrastructure, Grant/Award Number: ANR-10-INBS-04; University of Washington, Grant/Award Number: Royalty Research Fund award A118261; New York University; National Institute of General Medical Sciences, Grant/Award Number: GM096032; ASSEMBLE; Eunice Kennedy Shriver National Institute of Child Health and Human Development, Grant/Award Number: HD084814; Division of Biological Infrastructure - NSF, Grant/Award Number: DBI-0939454 BEACON; Division of Integrative Organismal Systems - NSF, Grant/Award Number: IOS-1940743

Abstract

Many species in the tunicate family Molgulidae have independently lost their swimming larval form and instead develop as tailless, immotile larvae. These larvae do not develop structures that are essential for swimming such as the notochord, otolith, and tail muscles. However, little is known about neural development in these nonswimming larvae. Here, we studied the patterning of the Motor Ganglion (MG) of *Molgula occulta*, a nonswimming species. We found that spatial patterns of MG neuron regulators in this species are conserved, compared with species with swimming larvae, suggesting that the gene networks regulating their expression are intact despite the loss of swimming. However, expression of the key motor neuron regulatory gene *Ebf (Collier/Olf/EBF)* was reduced in the developing MG of *M. occulta* when compared with molgulid species with swimming larvae. This was corroborated by measuring allele-specific expression of *Ebf* in hybrid embryos from crosses of *M. occulta* with the swimming species *M. oculata*. Heterologous reporter construct assays in the model tunicate species *Ciona robusta* revealed a specific *cis*-regulatory sequence change that reduces expression of *Ebf* in the MG, but not in other cells. Taken together, these data suggest that MG neurons are still specified in *M. occulta* larvae, but their differentiation might be impaired due to reduction of *Ebf* expression levels.

KEYWORDS

Cis-regulatory changes, Collier/Olf/EBF, evolutionary loss, *Molgula*, motor neurons, tunicates

1 | INTRODUCTION

The evolution of animal body plans has occurred not only through evolutionary novelties, but also through losses both subtle and catastrophic (Albalat & Cañestro, 2016). Although they are defined primarily by the absence of structures, cell types, or genes, the evolutionary loss of these various units of selection can help illuminate their functions. For instance, identifying traits that are retained in one species but lost in a closely related species can reveal which are likely to be under purifying selection and provide a measure of their relative adaptive value under certain conditions, for example, loss of vision in cave-dwelling organisms (Porter & Crandall, 2003).

Some of the most prominent examples of extensive evolutionary losses come from the tunicates (Denoeud et al., 2010; Huber et al., 2000), marine filter-feeding organisms characterized by a protective layer, or "tunic," made mostly of cellulose (Sasakura, 2018). Phylogenomic analyses suggest that vertebrates are the sister group to the tunicates (Delsuc et al., 2006), but most tunicates have a distinct dispersal phase carried out by swimming "tadpole" type larvae that bear the typical chordate body plan: a rigid notochord flanked by striated paraxial muscles, controlled by a dorsal central nervous system. Some tunicate groups, such as the pelagic salps and pyrosomes, have completely lost this larval phase. In other groups, like the paraphyletic ascidians, the evolutionary loss of swimming larvae is an ongoing process and has affected specific aspects of larval development and behavior. This phenomenon is particularly prominent in molgulid tunicates, in which many species have independently lost larval structures important for swimming (Hadfield et al., 1995; Huber et al., 2000). The best studied of these is *Molgula occulta*, which gives rise to anural (tailless), nonswimming larvae that metamorphose into juveniles without going through the dispersal period of active swimming observed in urodele (tailed) species (Berrill, 1931).

Underlying this radically divergent, nonswimming larval form is the loss of morphogenesis and differentiation of certain cell types that are dispensable for nonswimming larva, like the notochord, tail muscles, or pigmented sensory organs (Jeffery & Swalla, 1991; Swalla & Jeffery, 1990, 1992). For instance, *M. occulta* larval muscle cells do not differentiate and the species has even lost certain genes encoding proteins specifically required for muscle function, like larval muscle actin (Kusakabe et al., 1996). Furthermore, larvae of *M. occulta* lack a pigmented otolith cell, important for gravity sensing (Jiang et al., 2005; Tsuda et al., 2003), due to the loss of genes encoding functional enzymes required for melanogenesis like Tyrosinase and Tyrosinase-related protein (Tyrp; Racioppi et al., 2017). Over 17 species with nonswimming larvae have been described in *Molgula*, and all 31 species in the closely related molgulid genera *Eugyra* and *Bostrichobranchus* appear to have nonswimming larvae. The vast majority of molgulids (>150 species) have not been studied at the larval stage, leaving the possibility that many more nonswimming larvae have independently evolved within the clade (Maliska et al., 2013; Shenkar et al., 2019).

Little is known about the extent of nervous system development in the larvae of *M. occulta* and other species with non-swimming larvae. The typical tunicate larva has a minimal nervous system dedicated to controlling its swimming and settlement behavior in response to sensory cues such as light, gravity, and mechanical stimuli (Jiang et al., 2005; Kourakis et al., 2019; Rudolf et al., 2018; Zega et al., 2006). The larval nervous system of the tunicate *Ciona intestinalis* has been completely mapped, revealing 177 neurons in a dorsal central nervous system (CNS) and 54 peripheral sensory neurons distributed throughout the epidermis (Ryan & Meinertzhagen, 2019; Ryan et al., 2016; Ryan et al., 2018). This minimal nervous system is largely identical in other *Ciona* species, particularly *Ciona robusta*, which has become a model for the study of chordate-specific principles of neuronal function and development (Nishino, 2018). Because *C. robusta* was only recently proposed to be a species distinct from *C. intestinalis* (Pennati et al., 2015), we refer to *Ciona* spp. as simply "*Ciona*" herein.

Given the evolutionary loss of other structures important for swimming, such as notochord, muscles, and otolith, we asked whether any neurodevelopmental processes have been lost during the evolution of *M. occulta*. To this end, we surveyed the development of the nervous system in *M. occulta* embryos, using species with swimming larvae as a basis for comparison. Using in situ hybridization, RNAseq data, and cross-species transgenic assays, we report that neurodevelopmental gene expression and patterning is unexpectedly conserved in *M. occulta* larvae. Most notably, the Motor Ganglion (MG), the CNS structure that controls the swimming movements of tailed larvae (Nishino et al., 2010) is also found in tailless larvae. However, we uncovered specific mutations in *cis*-regulatory elements that might underlie the reduced transcriptional activity of the key motor neuron transcription factor-coding gene *Ebf* (Catela et al., 2019; Kratsios et al., 2012), specifically in larval motor neurons. Nevertheless, unlike *Tyrosinase* or *Tyrp*, the *Ebf* gene has not become pseudogenized in *M. occulta*, likely due to its requirement for the specification of other neurons and cell types that are still important for the complete life cycle of this ascidian.

2 | METHODS

2.1 | *Molgula* spp. embryo collection

Gonads were dissected from gravid *M. occulta* and *M. oculata* adults that were collected in August (the only time of the year when gravid individuals can be obtained) at Station Biologique in Roscoff, France. *M. occidentalis* adults were collected and shipped by Gulf Specimen Marine Lab (Panacea, FL). Eggs from dissected gonads were fertilized in vitro, dechorionated, and fixed as previously described (Stolfi et al., 2014).

2.2 | mRNA probe synthesis, in situ hybridization, and immunostaining

Templates for mRNA in situ hybridization probes were cloned by polymerase chain reaction or SMARTer 3′/5′-RACE (Clontech) from complementary DNA or genomic DNA, or synthesized de novo (Twist Bioscience). See Supporting Information for detailed sequences and sequence IDs to search for further information in the ANISEED database (Dardaillon et al., 2020). In vitro transcription of labeled probes for fluorescent in situ hybridization was performed as previously described (Ikuta & Saiga, 2007; Stolfi et al., 2014). To detect cilia, larvae were incubated with anti-acetylated α-tubulin primary antibody (clone 6-11B-1, Sigma) and AlexaFluor-488 secondary antibody (Thermo Fisher Scientific) as previously described (Pennati et al., 2015). Cell outlines were counterstained with phalloidin AlexFluor-546 conjugate (Thermo Fisher Scientific) incubated 1:50 for at least 2 h before final washes, and nuclei were labeled with 4′,6-diamidino-2-phenylindole during the final wash. Embryos were imaged on upright and inverted epifluorescence microscopes (Leica), or TCS SP5 AOBS and TCS SP8 X scanning point confocal systems (Leica).

2.3 | Hybrid embryo allele-specific differential expression analyses

M. occulta and *M. oculata* genomes (Stolfi et al., 2014) were improved using Redundans (Pryszcz & Gabaldón, 2016). Reads from the initial assemblies located on NCBI (PRJNA253689) were used to improve scaffolding in the *M. occidentalis* genome and in gene models we previously published (Lowe & Stolfi, 2018). In addition, the *M. oculata* genome was used as a reference to increase the scaffolding of the *M. occulta* genome. To build gene models, previously sequenced RNAseq reads (SRA accession number SRP040134; Lowe et al., 2014) were mapped using hisat2 (v2.1.0; Kim et al., 2015), converted to BAM, then sorted using SAMTools (v1.5; Li et al., 2009) and merged with picard tools (v2.0.1; http://broadinstitute.github.io/picard/). Merged bam files were assembled using Trinity genome-guide command (v2.6.6; Grabherr et al., 2011). Assembled transcripts were translated and filtered using TransDecoder (https://github.com/TransDecoder/TransDecoder/wiki) removing any open-reading frames less than 100 amino acids. To improve on contiguity, transcripts were BLASTed against *Ciona robusta* predicted proteins from ANISEED (Dardaillon et al., 2020), then scaffolded using TransPS (Liu et al., 2014). Default parameters used for all steps and scripts can be found at https://github.com/elijahlowe/tailed. Genomes and transcriptomes used in this study can be found at https://osf.io/mj3r7/. For differential expression analysis, previously sequenced RNAseq read fragments from *M. occulta, M. oculata* and *M. occulta* × *M. oculata* hybrid embryos from three different stages (Lowe, Fodor et al. in preparation; Lowe et al., 2014) were mapped to the *M. occulta* or *M. oculata Ebf* gene models using bowtie2 (v2.3.2; Langmead & Salzberg, 2012) and eXpress (v1.4.1; Roberts & Pachter, 2013) for quantification. Reads from hybrids were mapped onto both parent transcriptomes in the same manner to quantify allele specific expression. EdgeR (Robinson et al., 2010) was used to identify differentially expressed genes.

2.4 | Reporter construct cloning and electroporation into *Ciona robusta*

Ebf upstream *cis*-regulatory sequences were cloned from *M. occulta* and *M. oculata* genomic DNA. Mutations to convert *occulta* sequences to *oculata*-like sequences and vice-versa were generated by synthesizing DNA fragments (Twist Bioscience) and subcloning into the full-length reporter plasmids. See Supporting Information for all sequences. *C. robusta* (a.k.a. *intestinalis* Type A; Pennati et al., 2015) adults were collected and shipped by M-REP. Eggs were obtained from dissected adults and dechorionated, fertilized, electroporated, and fixed as previously described (Christiaen et al., 2009a; 2009b; Stolfi et al., 2014).

3 | RESULTS

3.1 | The nervous system of *M. occulta* larvae

M. occulta (Figure 1a) and *M. oculata* (Figure 1b) are closely related species that occur sympatrically off the coast of Brittany, France. In spite of their close genetic

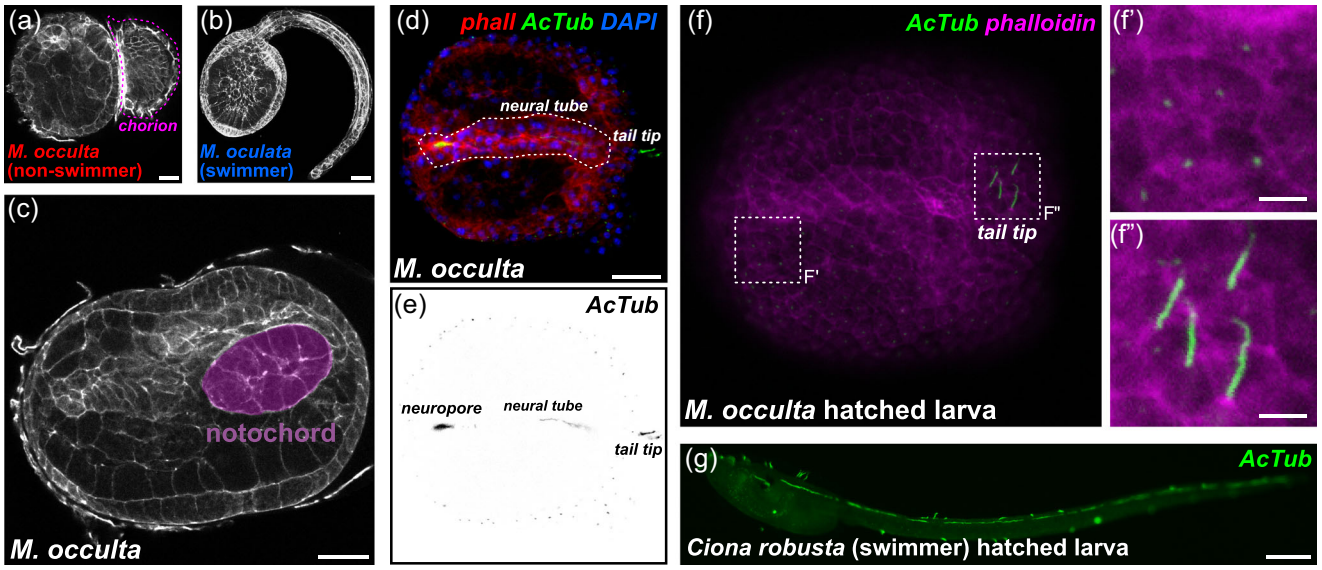

FIGURE 1 Development and morphology of the non-swimming larvae of *Molgula occulta* and *Molgula oculata*. (a) Hatching larva of nonswimming species *M. occulta*, squeezing out of the chorion (pink dotted outline). (b) Hatched larva of swimming species *M. oculata*. (c) Hatching *M. occulta* larva showing twenty notochord cells (purple) that fail to undergo convergent extension. Larvae in (a–c) stained with phalloidin conjugate. (d) *M. occulta* larva stained with phalloidin conjugate (phall, red), anti-acetylated alpha tubulin antibody (AcTub, green), and DAPI (blue), showing presence of acetylated alpha tubulin-rich cilia lining the neuropore/stomodeum and neural tube lumen, and tail tip epidermal cells. (e) Inverted monochrome image of acetylated alpha tubulin stain in (d). (f) *M. occulta* larva stained with anti-acetylated alpha tubulin antibody (AcTub, green) and phalloidin conjugate (magenta). Inset in (f') shows short cilia of epidermal cells covering the majority of the larva. Inset in (f") shows longer cilia (presumably sensory) of four tail tip cells. (g) Swimming larva of *Ciona robusta* stained with same anti-acetylated alpha tubulin antibody (AcTub, green), showing abundance of ciliated epidermal sensory cells and neural tube ependymal cells along the entire length of the larva including tail. Scale bars in (a–d): 25 μm. Scale bars in (f): 5 μm. Scale bar in (g): 75 μm. Anterior to the left in all panels. DAPI, 4′,6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

similarity and their ability to form interspecific hybrids (Swalla & Jeffery, 1990), the larvae of *M. occulta* are tailless and nonswimming (Figure 1c). Given its inability to swim, we asked whether the *M. occulta* larva has a functional nervous system or whether it has been partially lost in evolution like its vestigial notochord, muscles, and pigment cells. Confocal imaging of phalloidin-stained hatching *M. occulta* larvae revealed that they possess a dorsal hollow neural tube, though it is shortened along the anterior–posterior (A–P) axis due to the lack of an extended tail (Figure 1d). Acetylated tubulin immunohistochemistry revealed ciliated cells lining the neuropore and the shortened caudal nerve cord (Figure 1e). In the epidermis, we found that only four cells at the very posterior tip of the tail bore long cilia typical of tunicate epidermal sensory cells (Figure 1f). In contrast, swimming larvae like those of *C. robusta* (Figure 1g) have an extended caudal nerve cord lined with numerous ciliated ependymal cells down the length of the tail, and bear numerous ciliated sensory neurons scattered throughout the epidermis of the head and tail (Pasini et al., 2006; Ryan et al., 2018; Torrence & Cloney, 1982; Vorontsova et al., 1997). Thus, the shorter nerve cord and lack of epidermal sensory neurons (except for 4 tail-tip neurons) compared with swimming species' larvae suggest that larval nervous system development is incomplete in *M. occulta*.

Unfortunately, we were unable to visualize *M. occulta* neurons in greater detail using fluorescent reporter plasmids, as we have done in *Ciona* and *M. occidentalis* (Lowe & Stolfi, 2018; Stolfi & Levine, 2011). This is because *M. occulta* eggs are extremely difficult to fully dechorionate and electroporate. Although we previously reported obtaining electroporated *M. occulta* embryos (Stolfi et al., 2014), our low success rate and the limited geographic range and spawning season of *M. occulta/oculata* means that we have yet to perform routine transfection of these species. Under these conditions, we proceeded with our analysis using mainly in situ hybridization and next-generation sequencing in *M. occulta* embryos, and heterologous reporter construct assays in *C. robusta* embryos.

3.2 | Gene expression patterns in the *M. occidentalis* motor ganglion

In *Ciona*, the swimming and escape-response behaviors of the larva are controlled by the MG, a central pattern

generator comprised of 22 total neurons (Nishino et al., 2010; Ryan et al., 2016). Within the MG, there is a core of eight left/right pairs of neurons that form the bulk of the synaptic connections within the MG, as well as the majority of neuromuscular synapses (Ryan et al., 2016). We refer to the core MG neurons on only one side of the larva, with the implicit understanding that our discussion encompasses each left/right pair. We have previously characterized the core cell lineages of the MG in *Ciona* and *M. occidentalis*, documenting the highly conserved, invariant specification of each MG neuron subtype and their diagnostic marker gene expression patterns (Figure 2a; Imai et al., 2009; Lowe & Stolfi, 2018; Stolfi & Levine, 2011). If the major function of the MG in tunicate larvae is to simply drive swimming behavior, it is likely of little adaptive value and therefore likely to be lost in a nonswimming larva like that of *M. occulta*. On the other hand, MG progenitors might also contribute to the adult nervous system, or differentiated MG neurons might be carrying out other functions beyond swimming, such as triggering metamorphosis.

In situ hybridization for the pan-neural gene *Celf3/4/5* (also known as *ETR-1*) (Figure 2b; Yagi & Makabe, 2001), allowed visualization of the entire developing nervous system of *M. occulta*. The MG was seen as a mass of *Celf3/4/5*+ cells posterior to the heavily stained brain and the more lightly stained "neck" region. This suggested that some degree of MG neuron specification occurs in *M. occulta* larvae. Therefore, we sought to further characterize the development of the MG using more restricted, subtype-specific markers known from previous studies in swimming species.

3.3 | Motor neurons

To ask whether larval motor neurons are specified in *M. occulta*, we performed in situ hybridization with *Mnx*, a conserved regulator of motor neuron fate (Ferrier et al., 2001) which in *Ciona* and *M. occidentalis* labels the two left/right pairs of major motor neurons of the MG: MN1 and MN2 (Imai et al., 2009; Lowe & Stolfi, 2018; Ryan et al., 2016). To our surprise, we detected exactly two left/right pairs of cells expressing *Mnx* in tailless *M. occulta* "tailbud" embryos (Figure 2c). This staining appeared identical to *Mnx* expression in *Ciona* and *M. occidentalis*, with a gap between the two cells on either side where Interneuron 2 (IN2) should be.

To possibly distinguish the two motor neuron subtypes, we did in situ hybridization for *Nk6* and *Islet*, markers specifically for MN1 and MN2 respectively. *Nk6* strongly labeled one cell on either side of the MG (Figure 2d), with weaker possibly transient expression in an adjacent, more posterior cell. This is similar to its expression in *Ciona* and *M. occidentalis*, in which *Nk6* is initially expressed in both MN1 and IN2 (albeit more

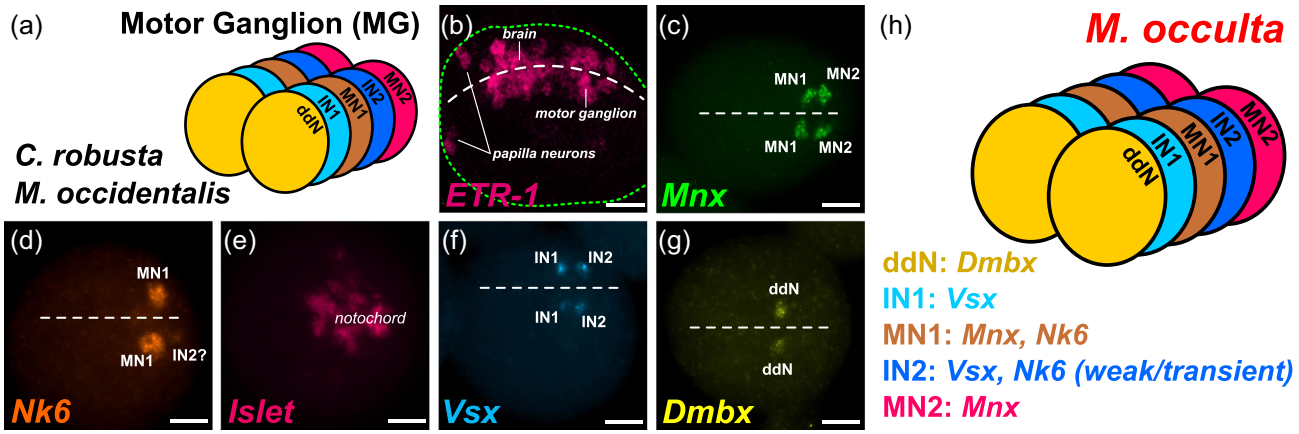

FIGURE 2 Gene expression patterns in the developing nervous system of *Molgula occulta*. (a) Summary of motor ganglion (MG) neuron subtype configuration described in swimming larvae of *Ciona robusta* and *M. occidentalis*. (b) Fluorescent whole mount in situ mRNA hybridization (ISH) in tailbud embryos for transcripts of neural marker gene *Celf3/4/5*, also known as *ETR-1*, showing the developing nervous system including brain, MG and papilla neurons. Embryonic dorsal midline indicated by white dashed line. Embryo outline indicated by green dashed line. (c) ISH for motor neuron-specific marker *Mnx*, showing expression marking cells on either side of the MG, separated by a single cell where it is not expressed. (d) ISH for *Nk6* (a marker of MN1 in late tailbud embryos of swimming species) showing expression in one or two adjacent cells on either side. (e) ISH for *Islet*, showing expression in disorganized notochord cells. (f) ISH for MG interneuron marker gene *Vsx*, showing expression in two cells on either side of the MG, also separated by a single nonexpressing cell. (g) ISH for descending decussating neuron (ddN)-specific marker *Dmbx*. Dorsal midlines indicated by dashed line. (h) Summary diagram of MG neuron subtype-specific gene expression in *M. occulta*. All scale bars: 25 μm. Anterior to the left in all panels [Color figure can be viewed at wileyonlinelibrary.com]

weakly) but is then strongly upregulated and maintained in only MN1 (Lowe & Stolfi, 2018; Stolfi & Levine, 2011). Thus, these cells in *M. occulta* appear to correspond to MN1 and IN2, based on their positions and the *M. occidentalis* pattern. In contrast, we were unable to clearly identify MN2 based on *Islet* expression, which was seen in an indistinguishable mass of cells in the middle of the embryo (Figure 2e). These cells likely correspond to the vestigial notochord, which expresses *Islet* in *M. occidentalis* and *Ciona* (Giuliano et al., 1998; Lowe & Stolfi, 2018). Although we were not able to visualize such clear *Islet* expression and assign an unambiguous MN2 identity to any neuron, we conclude that at least two left/right pairs of motor neurons are specified in *M. occulta* larvae, based on *Mnx* expression pattern.

3.4 | Interneurons

In the *Ciona* MG connectome, three pairs of descending interneurons are predicted to play an important role in both the rhythmicity of swimming movements and their modulation by inputs from the sensory vesicle (Kourakis et al., 2019; Ryan et al., 2016). The best studied of these interneurons are MGIN1 and MGIN2 (referred to from now on as IN1 and IN2 respectively), which flank MN1. Both are presumed excitatory interneurons that arise from the A9.30 lineage (Cole & Meinertzhagen, 2004). In *Ciona* and *M. occidentalis*, they are marked by expression of *Vsx* (Imai et al., 2009; Stolfi & Levine, 2011), the ortholog of conserved spinal cord interneuron regulator Vsx2/Chx10 (Altun-Gultekin et al., 2001; Kimura et al., 2006).

We found that in *M. occulta* embryos, *Vsx* labels two cells on either side of the neural tube at the tailbud stage (Figure 2f). This is similar to the orthologous expression patterns in *Ciona* and *M. occidentalis*. In *Ciona, Vsx* is activated in IN1 and IN2 at two different time points (Stolfi & Levine, 2011). IN2 is the first interneuron to differentiate, and activates *Vsx* early, before IN1 is even born. Later, after IN1 is born and specified, *Vsx* is activated in this cell. In *M. occidentalis*, we showed that *Vsx* expression appears concurrently in IN2 and in a progenitor cell that will give rise to IN1, indicating a temporal shift towards precocious activation of *Vsx* in the IN1 lineage (Lowe & Stolfi, 2018). Here, we were unable to ascertain the relative timing of *Vsx* expression and the identities of the *Vsx*-expressing cells but can conclude that at least two left/right pairs of *Vsx*+ interneurons are specified in *M. occulta*, likely corresponding to IN1 and IN2 in swimming species.

3.5 | Descending decussating neurons

The descending decussating neuron (ddN) is a highly unique neuron at the anterior of the core MG in both *Ciona* and *M. occidentalis*. It is the only descending neuron that decussates, or projects its axon contralaterally. Due to its position and connectivity within the *Ciona* MG, it was proposed to be the homolog of vertebrate Mauthner cells and to function in a homologous escape response pathway (Ryan et al., 2017; Takamura et al., 2010). In *Ciona* and *M. occidentalis*, the ddN is marked by expression of *Dmbx* (Lowe & Stolfi, 2018; Takahashi & Holland, 2004). In *M. occulta* tailbud embryos, *Dmbx* was found to be expressed in a single pair of cells (Figure 2g), likely corresponding to the ddNs or their immediate progenitors, the A11.120 cells. We conclude that the *M. occulta* larva specifies ddNs despite being physically incapable of engaging in escape response maneuvers.

Taken together, these data reveal that neuronal subtypes are likely arrayed in the *M. occulta* MG in a manner that is identical to that previously described in swimming species such as *M. occidentalis* and *Ciona* spp (compare Figure 2h with Figure 2a). We were not able to obtain enough *M. oculata* embryos for in situ hybridization to verify the expression patterns of all the genes described here, though expression patterns of *Mnx, Dmbx*, and *Onecut* in *M. oculata* suggested identical MG neuron distribution in this species as well (Supplementary Information). Regardless of whether each MG neuron subtype is in fact fully specified and differentiated in *M. occulta* as in swimming species, we show that fine-scale gene expression patterning of the *M. occulta* MG has not been evolutionarily lost.

3.6 | Reduced expression of *Ebf* as a potential mechanism of impaired MG neuron differentiation

Although the spatial patterning of the MG appeared to still be present in *M. occulta*, we further pursued the hypothesis that neuronal differentiation might not proceed in *M. occulta* embryos, similar to how the differentiation (but not the specification) of notochord, tail muscle, and otolith cells have been lost. We compared the expression of genes encoding major regulators of neurogenesis and neural specification, namely *Neurogenin, Onecut* (Racioppi et al., 2017) and *Ebf*, all previously shown to be required for neuronal differentiation in swimming tunicate larvae (Figure 3a–c; D'Aniello et al., 2011; Imai et al., 2009; Lowe & Stolfi, 2018; Satou et al., 2001; Stolfi et al., 2015). By in situ hybridization, all three were expressed in brain and MG progenitors, but *Ebf* in particular appeared substantially weaker in the

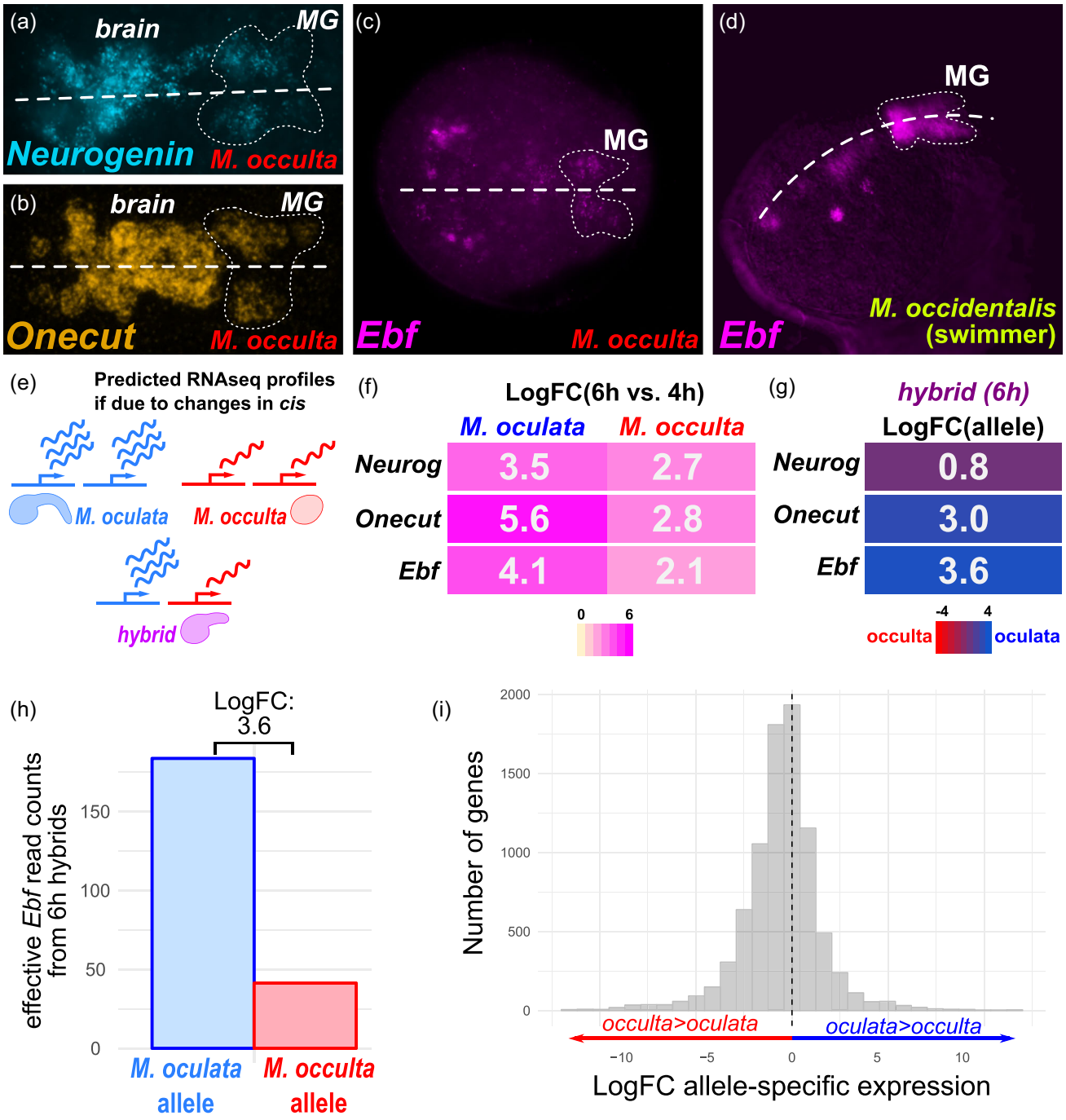

FIGURE 3 Loss of motor ganglion (MG)-specific transcriptional activity of the terminal selector gene *Ebf* in *Molgula occulta*. (a) Fluorescent whole mount in situ mRNA hybridization (ISH) for transcripts of the proneural bHLH gene *Neurogenin*, showing brain and motor ganglion (MG)-specific expression. MG cells outlined by dotted line. (b) ISH for neural regulatory gene *Onecut*, showing brain and MG-specific expression. MG cells outlined by dotted line. (c) ISH for *Ebf* in tailbud-stage *M. occulta* embryo. MG outlined by dotted line, showing relatively weak expression of *Ebf*. (d) ISH for *Ebf* in tailbud stage embryo of the swimming species *Molgula occidentalis*, showing strong expression in the MG (dotted outline). (e) Diagram of RNAseq analysis of hybrid embryos, produced by fertilizing eggs of nonswimmer *M. occulta* with sperm from swimmer *M. oculata* (left), and predicted allele-specific expression if reduced expression in *M. occulta* is due to changes in *cis*. (f) Table showing differential expression of selected genes in *M. oculata* and *M. occulta*, from neurula (4 h) to tailbud (6 h). (g) Table showing differential allele-specific expression of parental alleles in interspecific hybrids at 6 h. (h) RNAseq read counts from hybrid embryos at 6 h postfertilization (hpf), showing greater effective read counts from the *M. oculata Ebf* allele relative to the *M. occulta* allele. "Effective read counts" is the number of fragments estimated to arise from *Ebf*, adjusted for fragment and length bias, and is the direct input for our differential expression analysis. (e) Distributions of allele-specific expression measurements of genes in hybrids at 6 h binned by Log_2FC, showing no genome-wide bias towards *M. oculata* allele-specific expression. Positive values indicate more abundant *M. oculata* parental allele transcripts, negative values indicate more abundant *M. occulta* parental allele transcripts. Dorsal midline in (a–d) indicated by dashed line. Anterior to the left in (a–d) [Color figure can be viewed at wileyonlinelibrary.com]

MG (Figure 3c) compared with its orthologous expression in *M. occidentalis* (Figure 3d; Lowe & Stolfi, 2018), although this assay was not quantitative.

To further compare species-specific transcript abundance and look for potential differences in *cis*-regulatory activity, we analyzed RNAseq data from embryos of *M. occulta*, the closely related swimming species *M. oculata*, and their interspecific hybrids (*M. occulta* egg fertilized with *M. oculata* sperm; Figure 3e; Lowe, Fodor et al., in preparation). This particular cross results in only partial rescue of tailed larval structures, potentially revealing zygotic gene expression that may be crucial for the swimming larva phenotype (Swalla & Jeffery, 1990). In contrast, the converse (*M. oculata* egg fertilized with *M. occulta* sperm) results in fully tailed larvae (Swalla & Jeffery, 1990). In *M. oculata, Neurogenin, Ebf*, and *Onecut*, are upregulated at 6 h (tailbud stage), but in *M. occulta*, fold-change calculations were consistently lower than in *M. oculata* (Figure 3f). When we mapped RNAseq reads obtained from the interspecific hybrid to alleles from either parent, we observed differential allele-specific expression especially for *Ebf* and *Onecut*, less so for *Neurog* (Figure 3g). The depletion of *M. occulta Ebf* allele reads in the hybrid ($Log_2FC = -3.6$; Figure 3h) was especially interesting, given the reduced expression of this gene in *M. occulta* as assayed by either in situ hybridization or RNAseq. To verify whether this enrichment was due to an overall bias in the detection/mapping of *M. oculata* reads, we looked at all 2311 genes that showed allele-specific expression at 6 hpf (Figure 3i). 1292 (56%) of these 2311 genes actually showed less *M. oculata* parental allele expression relative to the *M. occulta* allele ($Log_2FC<0$). Thus, the enrichment of *M. oculata Ebf* allele expression in the hybrid might reflect changes in *cis* that underlie its reduced expression in the *M. occulta*. However, we were unable to process biological duplicates for most of these samples, which is why we decided to further investigate the activities of *cis*-regulatory sequences of *Ebf* from both species.

3.7 | A *cis*-regulatory change affecting *Ebf* expression specifically in MG neurons, but not in other cells

To test for potential differences in *cis* that could explain this differential activity of *M. occulta* and *M. oculata* parental *Ebf* alleles, we constructed reporter plasmids using *cis*-regulatory sequences upstream of *Molgula Ebf* genes. Unfortunately, given our inability to electroporate *M. occulta/oculata*, we turned to *C. robusta*, which can be routinely electroporated in the laboratory with plasmid DNA (Zeller, 2018). When we electroporated *M. oculata Ebf* reporter constructs (*oculata. Ebf-3654/+24>XFP*) into *Ciona* embryos, we observed reporter gene expression in MG neurons, bipolar tail neurons, and some brain neurons (Figure 4a). Although there is no noncoding

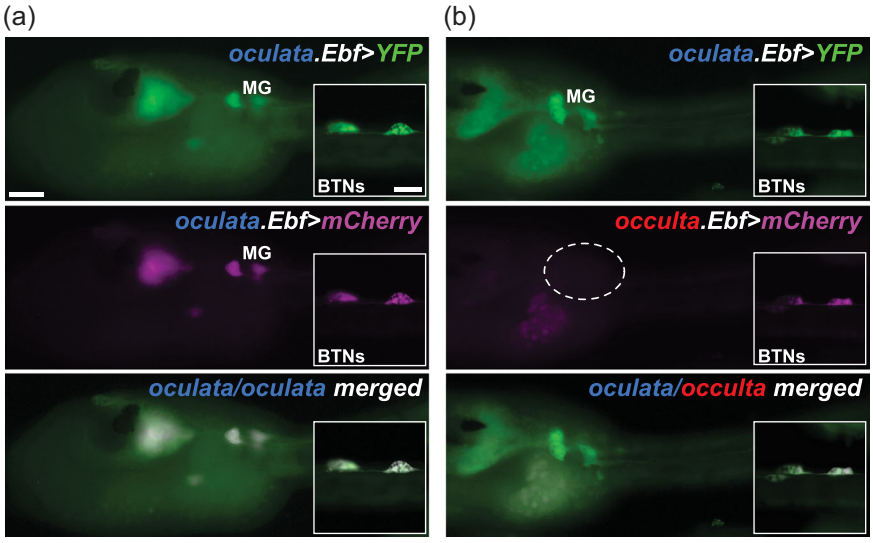

FIGURE 4 Differential activity of *Ebf* reporter plasmids in *Ciona robusta* electroporation assay. (a) A *Ciona robusta* larva electroporated with a mixture of *Molgula oculata Ebf>YFP* (green channel top) and *M. oculata Ebf>mCherry* (red channel middle) plasmids, showing perfect coexpression in the motor ganglion (MG), and in other neurons including bipolar tail neurons (BTNs, insets), merged in bottom panel. (b) Another *C. robusta* larva electroporated with a mixture of *M. oculata Ebf>YFP* (green channel top) and *M. occulta Ebf>mCherry* (red channel middle) plasmids, showing absence of mCherry expression specifically in the MG (region indicated by dashed circle), merged in bottom panel. In contrast, co-expression of YFP and mCherry in BTNs (insets) shows that reporter plasmid activity in other neurons is conserved. Scale bars in (a): 25 μm. All panels and insets are at the same respective scales. Anterior to the left in all panels [Color figure can be viewed at wileyonlinelibrary.com]

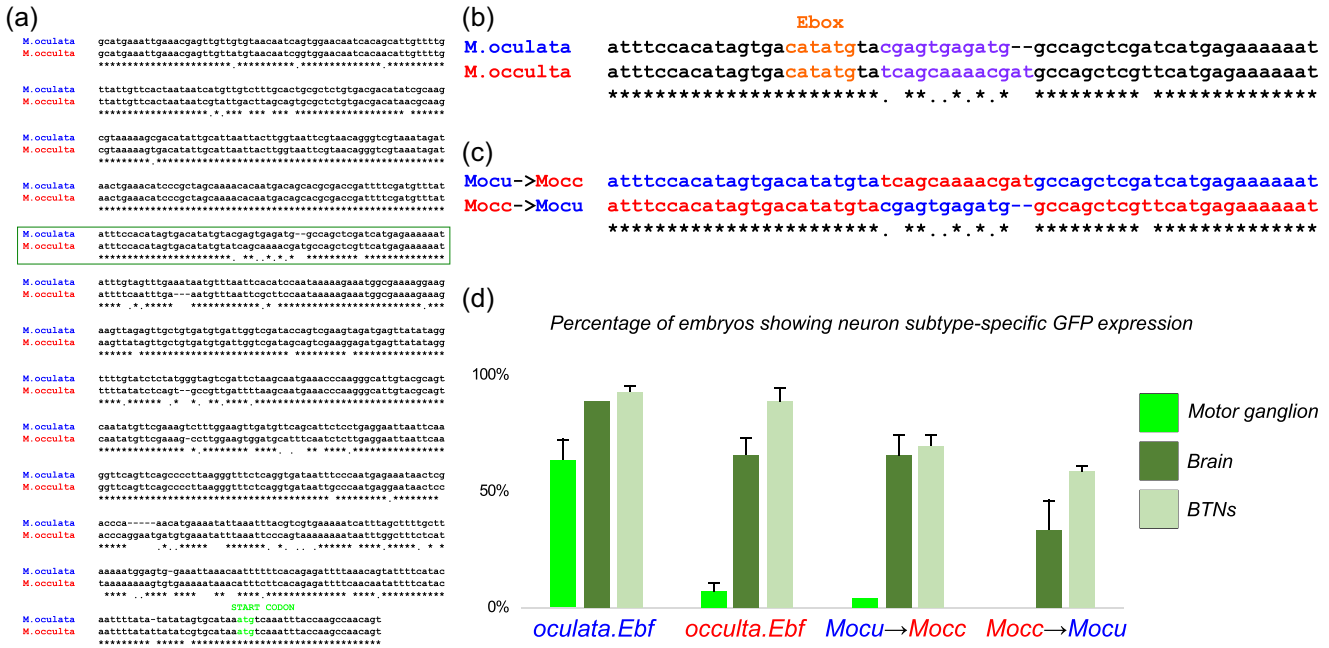

FIGURE 5 A *cis*-regulatory change underlying motor ganglion (MG)-specific loss of *Ebf* expression in *Molgula occulta*. (a) Alignment of sequences 5′ upstream of *Ebf* in *M. oculata* and *M. occulta*. (b) Sequence from boxed area in A, showing highly conserved sequences including a candidate E-box site potentially bound by Neurogenin, adjacent to a highly divergent motif (purple font). (c) Mutated sequences in *Mocu → Mocc > GFP* and *Mocc → Mocu > GFP* constructs in which the highly divergent motif has been swapped between *M. oculata* and *M. occulta Ebf* reporter constructs. (d) Results of scoring *Ciona robusta* larvae electroporated with the wild-type parental *Ebf* reporter plasmids and the mutated "swapped" constructs. Changing the *M. oculata* divergent motif sequence to the *M. occulta* sequence results in loss of GFP expression specifically in the motor ganglion, but not in brain or bipolar tail neurons (BTNs). The converse mutation, changing the *M. occulta* motif to resemble the *M. oculata* sequence was not sufficient to restore GFP expression in the motor ganglion. $n = 50$ embryos for each construct per replicate. Two replicates were performed with each construct. Bars indicate average percentages for each construct, errors bars indicate range [Color figure can be viewed at wileyonlinelibrary.com]

sequence conservation between *Molgula* and *Ciona*, and acute developmental system drift has rendered many *Molgula cis*-regulatory sequences inactive in *Ciona* (Stolfi et al., 2014), in this case there was enough conservation of the underlying regulatory logic to recapitulate the expression of *Ebf* in these various *Ciona* neuronal subtypes.

When we electroporated the corresponding *M. occulta Ebf* reporter constructs (*occulta. Ebf-3659/+24>XFP*), reporter gene expression was observed in all the same neurons *except* MG neurons (Figure 4b). Thus, our heterologous reporter plasmid assays in *Ciona* indicated that *M. occulta*-specific *cis*-regulatory changes might underlie lower levels of *Ebf* expression in MG neurons, without affecting its expression in other neurons.

To identify the potential *cis*-regulatory changes underlying this difference in *Ebf* activation, we aligned 735 bp of noncoding sequence immediately 5′ of *M. oculata Ebf* start codon to the orthologous sequence in *M. occulta* (Figure 5a). The aligned sequences share more than 90% identity, consistent with the close genetic relationship between these two species. This alignment revealed an unusually divergent sequence motif approximately 470 bp upstream of the *Ebf* translation start codon (Figure 5b). This sequence was of particular interest to us because these changes were observed adjacent to a conserved E-box site that might be a binding site for Neurogenin. In *C. robusta, Ebf* is activated in the MG by the proneural bHLH factor Neurogenin (Imai et al., 2009). We also identified E-box sites in experimentally validated upstream *Ebf* cis-regulatory sequences isolated from *C. robusta, C. savignyi*, and *M. occidentalis* (Supporting Information), even though these sequences show poor overall conservation with the *M. oculata/occulta* sequences. The E-box site is conserved between the two *Ciona* species, further supporting the hypothesis that this site is important for *Ebf* activation. The presence of an E-box within approximately 800 bp of the start codon in all tunicate species surveyed suggested this region might be part of a functionally conserved minimal enhancer for MG-specific *Ebf* regulation (Racioppi et al., 2019). When we compared all three *Molgula* sequences, we found that this E-box-adjacent sequence (AGATGGC) was conserved in both *M. oculata* and the more distantly related *M. occidentalis*, but not in *M. occulta*. Because the larvae of *M. occidentalis* are also swimming, and because

this *M. occidentalis Ebf* cis-regulatory sequence also drives reporter gene expression in *C. robusta* MG neurons (Stolfi et al., 2014), we hypothesized that the E-box-adjacent sequence may be crucial for MG-specific expression.

To directly test whether modifications to the sequences adjacent to the E-box might explain the different transcriptional activities of swimming versus nonswimming *Molgula Ebf* loci, we introduced point mutations in the E-box-adjacent sequence of *oculata.Ebf-3654/+24>GFP* to match the *M. occulta* sequence (CGAGTGAGATG to TCAGCAAAACGAT; Figure 5c). When we electroporated this mutated reporter plasmid (*Mocu–>Mocc>GFP*) in *Ciona*, we found that its transcriptional activity was almost completely abolished specifically in the MG, but had little effect on reporter gene expression in brain or bipolar tail neurons (Figure 5d). Although we attempted to "resurrect" the putative ancestral sequence by mutating the E-box-adjacent site in *occulta.Ebf-3659/+24>GFP* to resemble the *M. oculata* sequence (*Mocc–>Mocu>GFP)*, this was not sufficient to restore activity, hinting at the accumulation of multiple loss-of-function mutations in other parts of the *M. occulta Ebf cis*-regulatory sequence.

These data show that, when tested in *Ciona*, the E-box-adjacent sequence is necessary for the MG-specific activity of *M. oculata Ebf cis*-regulatory activity. Alternatively, the *M. occulta*-specific sequence might have created a novel binding site for a MG-specific repressor, but the inability of the *Mocc– > Mocu* mutation to restore activity does not support this hypothesis. In sum, although we have identified the loss of the E-box adjacent sequence as one difference underlying the differential activity of these species' *cis*-regulatory sequences, the *M. occulta* regulatory sequences have likely accumulated many more differences.

4 | DISCUSSION

We have investigated the molecular signatures of MG neuron specification and differentiation in a nonswimming molgulid tunicate larva, finding a surprising conservation of cell-specific expression patterns of key regulators of differentiated neurons (e.g., *Mnx, Dmbx*, and *Vsx*) during MG development. This indicates that some MG neurons are specified in *M. occulta* larvae, in spite of the evolutionary loss of the larval tail and swimming behavior. Unfortunately, we were unable to investigate neuronal morphology using reporter plasmids, due to our inability to routinely transfect *M. occulta* embryos. Once this method is established, it will be interesting to revisit this question to fully document the extent of nervous system and MG development in this species.

Using RNAseq, heterologous reporter plasmid assays, and *cis*-regulatory functional analyses, we demonstrated that specific changes to conserved *cis*-regulatory sequences upstream of *M. occulta Ebf* have likely altered its expression in MG neurons, but not in other neurons or cell types of *M. occulta*. What might be the exact nature of these *cis*-regulatory changes? Using the transcription factor DNA-binding preference database JASPAR (Khan et al., 2017), we found that the *M. oculata* E-box-adjacent sequence resembles a GATA-binding site (see Table S1). A similar GATA-binding site was also found adjacent to an E-box site in *C. robusta* and *C. savignyi Ebf cis*-regulatory sequences (see Table S1). Gata.b, one of two GATA orthologs in *Ciona*, is broadly expressed in the central nervous system at the tailbud stage (Imai et al., 2004), and could conceivably be acting as a cooperative binding partner of Neurogenin to activate transcription of *Ebf*. Curiously, the *M. occulta* sequence has not lost the predicted GATA-binding site, but its altered E-box-adjacent sequence contains a 3-bp insertion that shifts the putative GATA-binding site slightly further away from the E-box (Supporting Information). Given the importance of proper spacing in assuring spatiotemporally faithful transcriptional activation in *Ciona* development (Farley et al., 2015), perhaps the reduced activity of *M. occulta Ebf* expression in the MG is due in part to the increased spacing between these putative transcription factor binding sites. Alternatively, the divergent *M. occulta* sequence resembles a Sox1/2/3 binding site (see Table S1). In the *C. robusta* MG, *Sox1/2/3* suppresses MG neuron differentiation (Stolfi et al., 2011), as Sox2 does in the vertebrate spinal cord (Graham et al., 2003). Thus, a novel Sox1/2/3 binding site in this position might also underlie active repression of *Ebf* expression in *M. occulta*.

What might be the phenotypic consequences of lower *Ebf* transcription in *M. occulta* larval MG neurons? Ebf has been shown to regulate the terminal differentiation of motor neurons throughout Metazoa, including *C. elegans, Ciona*, and vertebrates (Catela et al., 2019; Kratsios et al., 2012; Velasco et al., 2017). In *C. elegans* motor neurons, Ebf was shown to be necessary for cholinergic gene expression, suggesting a role as a "terminal selector" for cholinergic motor neurons. In *Ciona*, ectopic *Ebf* overexpression is sufficient to activate a *Vesicular Acetylcholine Transporter (VAChT, or Slc18a3)/Choline Acetyltransferase (ChAT)* reporter in certain cell types, and an *Ebf* binding motif upstream of this cholinergic locus was identified bioinformatically, suggesting a conserved function in regulating cholinergic gene expression (Kratsios et al., 2012). However, requirement of Ebf for motor neuron-specific cholinergic gene expression was

previously tested by overexpression of a constitutive repressor form of Ebf (Ebf::WRPW; Kratsios et al., 2012), but not with a true *Ebf* loss-of-function condition, and *Ciona VAChT/ChAT* reporter constructs using shorter, more proximal *cis*-regulatory sequences lacking the *Ebf* binding motif are sufficient to drive reporter expression in cholinergic *motor neurons* (Yoshida et al., 2004). A recent study in mice showed that Ebf2 is required for MN differentiation, but not for expression of acetylcholine biosynthesis pathway genes (Catela et al., 2019). These findings suggest that, although EBF factors are important regulators of motor neuron fate and differentiation in tunicates and vertebrates, perhaps their direct regulation of cholinergic gene expression may be limited to certain neurons other than motor neurons.

One previously validated target of Ebf in *Ciona* MN2 is *Islet*, since CRISPR/Cas9-mediated knockout of *Ebf* results in loss of *Islet* expression in this neuron (Stolfi et al., 2014). We were unable to detect *Islet* expression in the *M. occulta* MG by in situ hybridization, but this may have been obscured by expression in the notochord (Figure 2d). Although this would be consistent with loss of Ebf-regulated *Islet* in the MG, these changes are hard to interpret in light of the abundant expression of *Islet* in the notochord. Thus, to fully understand the effects of loss of *Ebf* expression in the MG of *M. occulta*, more work will be needed to characterize the transcriptional targets of this important regulator in tailed, swimming tunicate larvae.

Ebf represents a distinct example of tissue-specific gene expression loss in *M. occulta* evolution. Previously, it has been shown that evolutionary loss of functional larval structures in *M. occulta* correlates with loss-of-function, protein-coding mutations resulting in the loss of crucial proteins such as muscle actin for tail muscle activity (Kusakabe et al., 1996) or Tyrosinase for melanin pigment synthesis in the gravity-sensing otolith organ (Racioppi et al., 2017). However, these proteins are only expressed in larval structures involved in larval motility (tail muscle cells and otolith, respectively), being apparently dispensable for the development of other larval and adult tissues. In contrast, *M. occulta Ebf* is not a pseudogene and is predicted to encode a fully functional protein. This difference is likely because Ebf is a regulator that may be required for the development of myriad cell types throughout the life cycle of *M. occulta*, especially in the sessile adults of this species, which are morphologically similar to adults of other species, including those with swimming larvae. Thus, even if *Ebf* expression is not needed in MG motor neurons (in the absence of functional larval muscles), its requirement for other processes in tunicate development (Racioppi et al., 2019; Razy-Krajka et al., 2014) may have resulted in the evolutionary loss of tissue-specific *cis*-regulatory elements, as our data indicate. It may be that the mechanisms we show here underlying cell-specific loss of *Ebf* expression in *M. occulta* are representative of constraints acting on traits that are lost from a particular developmental stage, but that are still retained in a different stage of the same organism's life cycle. Studying these losses and the mechanisms underlying such losses will complement the principles learned from studies of traits that are dispensable to the entire life cycle of a particular species, such as the loss of vision in cave-dwelling organisms.

Is the evolutionary loss of MG development an ongoing process in *M. occulta*? Might these processes break down further, as has happened with the loss of genes encoding melanogenesis or muscle structural proteins in this same species? Has MG neuron subtype-specific gene expression persisted because the same regulatory logic is required for expression in adult neurons or other tissues? Are there other constraints that have maintained the neuron subtype-specific marker gene expression we see in the MG? We know that some MG precursors remain undifferentiated in the larva but can contribute to the adult nervous system (Horie et al., 2011). Perhaps the cell-specific transcription factor expression we observe in the *M. occulta* MG reflects shared mechanisms of patterning both differentiated larval neurons and undifferentiated adult neural precursors. Another intriguing possibility is that the tunicate MG is required for other larval behaviors beyond swimming. For instance, perhaps MG neurons are required for triggering developmental processes during the metamorphosis of the larva into a juvenile. In this scenario, specification of distinct MG neuron subtypes might still be necessary to trigger metamorphosis of *M. occulta* larvae, even if Ebf -dependent functions in these neurons are not required and might have even been selected against. A deeper understanding of the mechanisms underlying both larval and adult neurogenesis in tunicates, and the role of neural activity in the transition to the adult phase, will be needed to answer these and other questions related to the loss of larval-specific structures in nonswimming species.

ACKNOWLEDGMENTS

We dedicate this manuscript to the memory of Alexander "Zander" Fodor. We thank C. Titus Brown for his encouragement and continued support. We thank Stéphane Hourdez, Sophie Booker, and Xavier Bailly of the Station Biologique for help in carrying out experiments at Roscoff. We thank Susanne Gibboney for assistance with cloning *Molgula Ebf* reporter plasmids. We thank Lindsay Turner for critical reading of the manuscript. This study was supported by NIH award R00 HD084814 and

NSF award IOS-1940743 to AS, by start-up package from the College or Arts and Science at New York University and NIH award R01 GM096032 to LC, by France BioImaging infrastructure ANR-10-INBS-04 to NP, and by a University of Washington Royalty Research Fund award (A118261) to BJS. The collaborative MoEvoDevo network is supported by ASSEMBLE (Association of European Marine Biological Laboratories) MARINE. CR was supported by a long-term fellowship ALTF 1608-2014 from EMBO and by a travel grant from the Boehringer Ingelheim Fonds. This material is also based in part upon collaborative work by BJS and EKL and C. Titus Brown, supported by the National Science Foundation under Cooperative Agreement No. DBI-0939454 BEACON, A Center for the Study of Evolution in Action. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Elijah K. Lowe, Nadine Peyriéras, Filomena Ristoratore, Lionel Christiaen, Billie J. Swalla, and Alberto Stolfi designed and supervised the experiments. Elijah K. Lowe, Claudia Racioppi, Nadine Peyriéras, Lionel Christiaen, Billie J. Swalla, and Alberto Stolfi handled the specimens and performed the experiments. Elijah K. Lowe performed bioinformatic data analysis. Elijah K. Lowe, Nadine Peyriéras, Filomena Ristoratore, Lionel Christiaen, Billie J. Swalla, and Alberto Stolfi supervised data collection and interpretation. Nadine Peyriéras, Filomena Ristoratore, Lionel Christiaen, Billie J. Swalla, and Alberto Stolfi secured funding for the study. All authors contributed to writing and editing the paper.

DATA AVAILABILITY STATEMENT

Previously published raw sequencing reads are already available (SRA accession number SRP040134). All code and scripts used in this study can be found at https://github.com/elijahlowe/tailed All other data available upon request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Lowe EK, Racioppi C, Peyriéras N, et al. A *cis*-regulatory change underlying the motor neuron-specific loss of *Ebf* expression in immotile tunicate larvae. *Evolution & Development*. 2020;e12364. https://doi.org/10.1111/ede.12364