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Evolution of *Snail*-mediated regulation of neural crest and placodes from an ancient role in bilaterian neurogenesis

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ABSTRACT

A major challenge in vertebrate evolution is to identify the gene regulatory mechanisms that facilitated the origin of neural crest cells and placodes from ancestral precursors in invertebrates. Here, we show in lamprey, a primitively jawless vertebrate, that the transcription factor *Snail* is expressed simultaneously throughout the neural plate, neural plate border, and pre-placodal ectoderm in the early embryo and is then upregulated in the CNS throughout neurogenesis. Using CRISPR/Cas9-mediated genome editing, we demonstrate that *Snail* plays functional roles in all of these embryonic domains or their derivatives. We first show that *Snail* patterns the neural plate border by repressing lateral expansion of *Pax3/7* and activating *nMyc* and *ZicA*. We also present evidence that *Snail* is essential for *DlxB*-mediated establishment of the pre-placodal ectoderm but is not required for *SoxB1a* expression during formation of the neural plate proper. At later stages, *Snail* regulates formation of neural crest-derived and placode-derived PNS neurons and controls CNS neural differentiation in part by promoting cell survival. Taken together with established functions of invertebrate *Snail* genes, we identify a pan-bilaterian mechanism that extends to jawless vertebrates for regulating neurogenesis that is dependent on *Snail* transcription factors. We propose that ancestral vertebrates deployed an evolutionarily conserved *Snail* expression domain in the CNS and PNS for neurogenesis and then acquired derived functions in neural crest and placode development by recruitment of regulatory genes downstream of neuroectodermal *Snail* activity. Our results suggest that *Snail* regulatory mechanisms in vertebrate novelties such as the neural crest and placodes may have emerged from neurogenic roles that originated early in bilaterian evolution.

1. Introduction

The origin of the vertebrates has been one of the most important and controversial topics in evolutionary biology and natural history for almost 200 years (Gegenbaur, 1878; Geoffroy Saint-Hilaire, 1830; Haeckel, 1860; Romer, 1950). Much of what distinguishes the vertebrates from their invertebrate relatives can be traced to two small cell populations, neural crest and placodes, which appear only transiently in vertebrate embryos (Gans and Northcutt, 1983; Gee, 1996; Green et al., 2015; Horstadius, 1950; Le Douarin and Kalcheim, 1999; Northcutt and Gans, 1983). The neural crest is a migratory, multipotent cell population that forms along the dorsal neural tube, from which it then detaches, migrates and differentiates into a wide array of cell types, including craniofacial cartilage and bone, smooth muscle, pigment, as well as most of the neurons and glia of the peripheral sensory nervous system (Green et al., 2015; Simões-Costa and Bronner, 2015). Placodes, which arise as ectodermal thickenings in the head, also migrate and give rise to cells in special sense organs (e.g., lens, otic, nasal, adenohypophysis, lateral line) as well as many of the sensory neurons in cranial ganglia (Graham and Shimeld, 2013; Schlosser, 2010, 2014, 2015). Both cell populations are tightly regulated during development by evolutionarily conserved gene regulatory networks (GRNs) that involve the activity of numerous signaling molecules and transcription factors (Betancur et al., 2010; Maharana and Schlosser, 2018; Sauka-Spengler and Bronner-Fraser, 2008; Sauka-Spengler et al., 2007; Schlosser, 2006). Together, neural crest and placodes form a wide range of vertebrate novelties and are therefore thought to have driven the origin and diversification of the vertebrate body plan (Gans and Northcutt, 1983; Northcutt, 2005; Northcutt and Gans, 1983; Trainor, 2013).

Despite their importance in vertebrate development, the evolutionary origins of neural crest and placodes, as well as their underlying regulatory networks, have proven enigmatic. An example of this is highlighted by vertebrate *Snail* genes (*Snail1, Snail2*, also known as *Snai1* and *Snai2*), which belong to the *Snail* superfamily, a group of zinc finger transcription

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factors with deep phylogenetic origins (Barrallo-Gimeno and Nieto, 2005, 2009; Grau et al., 1984; Manzanares et al., 2001; Nieto, 2002; Nieto et al., 1994; Nüsslein-Volhard et al., 1984). *Snail* genes likely duplicated from a single ancestral gene present in the last common ancestor of eumetazoans and have since acquired a diverse repertoire of important functions throughout eumetazoan embryogenesis (Barrallo--Gimeno and Nieto, 2009; Hemavathy et al., 2000; Nieto, 2002; Wu and Zhou, 2010). Among eumetazoans, *Snail1* and *Snail2* are pivotal for mesodermal genesis and patterning (Barrallo-Gimeno and Nieto, 2009; Nieto, 2002). However, they also control development of neural crest and placodes during various stages of vertebrate embryogenesis (Locascio et al., 2002; Manzanares et al., 2001; Nieto, 2001, 2002; Taneyhill et al., 2007). This suggests that *Snail*-mediated regulation of neural crest and placodes could have been co-opted from a genetic program for mesoderm development (Langeland et al., 1998; Manzanares et al., 2001; Nieto, 2002). An alternative to co-option from a mesodermal regulatory program is the possibility that neural crest and placode regulation might have been acquired from a program driving neurogenesis. Support for this hypothesis comes from the fact that *Snail* is expressed in, and regulates the development of, central nervous system (CNS) neurons in diverse lophotrochozoans, ecdysozoans, and invertebrate deuterostomes (Ashraf and Ip, 2001; Barrallo-Gimeno and Nieto, 2009; Dill et al., 2007; Hudson et al., 2015; Kerner et al., 2009; Langeland et al., 1998; Lespinet et al., 2002; Manzanares et al., 2001; Nieto, 2002; Stollewerk, 2016). However, vertebrates do not use *Snail* to pattern their nervous system and invertebrates were thought to lack neural crest and placodes (Gans and Northcutt, 1983; Green et al., 2015; Northcutt and Gans, 1983). Thus, apparent similarities in the use of *Snail* in neural crest and placodes of vertebrates with a CNS-neurogenic function among invertebrates appear superficial and suggest that *Snail* regulatory activity in these different cell populations may have evolved independently.

However, recent work suggests that there may be a closer affinity than previously thought between *Snail* involvement in neurogenesis of invertebrates and in neural crest and placodes among vertebrates. Functional experiments and lineage tracing in tunicates–now recognized as the closest living relatives of vertebrates–suggest that these animals may have embryonic rudiments of both neural crest and placodes, and the regions that generate these rudiments express *Snail* similar to vertebrate embryos (Abitua et al., 2012, 2015; Delsuc et al., 2006; Horie et al., 2018; Jeffery, 2006; Jeffery et al., 2004, 2008; Stolfi et al., 2015). Tunicates also use *Snail* to pattern the neural plate and CNS, a function similar to that among lophotrochozoans and ecdysozoans (Hudson et al., 2015, 2018). These observations suggest that *Snail* function in deuterostomes such as tunicates shares features with both protostome invertebrates on one hand (CNS, neurogenesis), and vertebrates on the other (neural crest, placodes). However, so far there has been no evidence of a vertebrate *Snail* gene that bridges the regulatory gap linking a CNS-neurogenic domain with neural crest and placodes across the invertebrate-vertebrate divide. In contrast to this, we and others have demonstrated that *Snail* is expressed throughout much of the developing CNS in embryos of the sea lamprey (*Petromyzon marinus*) (Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017). Lampreys are members of a primitively jawless group of vertebrates (cyclostomes) that are the sister group to all other (jawed, or gnathostome) vertebrates and therefore occupy a critical phylogenetic position to understand the earliest events in vertebrate history, including the origins of neural crest and placode regulation (Green and Bronner, 2014; McCauley et al., 2015; Rahimi et al., 2009; York et al., 2017, 2019). We previously showed that *Snail* expression and function in lampreys shares similarities with jawed vertebrates, including roles in neural crest migration and differentiation (Barrallo-Gimeno and Nieto, 2005; Dill et al., 2007; Lespinet et al., 2002; Nieto, 2002; Rahimi et al., 2009; Weller and Tautz, 2003; York et al., 2017). Despite these similarities, the broad neural expression in the CNS of lamprey *Snail* is unlike that of any other vertebrate but is similar to that in the developing nervous systems of protostome invertebrates, as well as invertebrate deuterostomes such as tunicates and amphioxus. However, the phylogenetic significance of these observations has not yet been explored.

Here, we address this issue by focusing on *Snail* expression and function in the CNS, neural crest, and placodes to gain insight into the potential roles of *Snail* in each of these populations in lamprey embryos. We then cast our results within a broad comparative embryology framework to test if the pre-vertebrate origins of *Snail* function in neurogenesis might be linked to the evolution of *Snail* regulation in neural crest and placodes in vertebrates. To this end, we describe lamprey *Snail* expression broadly in the neuroectoderm, encompassing the neural plate, neural plate border, and pre-placodal ectoderm contiguously, and show that *Snail* maintains robust expression in cells derived from these territories throughout embryogenesis. Using CRISPR/Cas9-mediated genome editing, we find that while *Snail* is not essential for early development of the neural plate, it is required for development of the neural plate border, in part by activating gene expression, but also by patterning the medial-lateral axis of the neural plate border. We also demonstrate a role for *Snail* in establishment of the pre-placodal ectoderm, and in formation of cranial sensory neurons of both placode and neural crest origin. After confirming a role in early neural crest and placode development, we finally demonstrate that although *Snail* is not essential for CNS neurogenesis, it is required for neurogenic differentiation within the CNS and does so by promoting cell survival. Taken together, our results demonstrate that lamprey *Snail* is expressed in, and appears to be capable of, regulating the development of neural crest, placodes, and CNS neurons simultaneously. This multi-functional role shares similarities with both vertebrates (neural crest, placode development) and invertebrates (CNS and PNS neurogenesis). We propose that these expression domains and functional roles in lamprey reflect an ancestral *Snail*-positive domain for neural crest and placode development in vertebrates that was co-opted from an evolutionarily ancient role in CNS and PNS neurogenesis that is conserved across bilaterians.

2. Materials and methods

2.1. Embryo collection and gene cloning

Gravid sea lampreys were collected from streams and rivers near the Hammond Bay Biological Station, Millersburg, MI, and shipped to the University of Oklahoma. Adult lampreys were maintained at 14 °C in a recirculating water system. Eggs were obtained manually from ovulating females and then mixed with sperm expressed from a mature male in a small beaker of water. Embryos were raised in Pyrex dishes under the flow of water (18 °C) that was supplemented with 0.05× Marc's Modified Ringers solution. All procedures requiring adult lampreys were approved by the University of Oklahoma Institutional Animal Care and Use Committee (IACUC, R15-027). Embryos were staged according to Tahara (1988), with those from desired stages for analysis fixed in MEMFA. *PCNA* and *Phox2* partial cDNA fragments were cloned from a sea lamprey cDNA library kindly provided by J. Langeland. Other partial cDNA clones (*nMyc*, *NCAM, ZicA*, *Tfap2a*, *Snail, SoxB1a, Pax3/7, DlxB, Six1/2*) were obtained from previous library screenings or PCR-based isolation as described elsewhere (McCauley and Bronner-Fraser, 2002, 2006; Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017).

2.2. In situ hybridization and immunohistochemistry

Protocols for *in situ* hybridization were performed as previously described (York et al., 2017). For immunohistochemistry involving Hu (Hu C/D, mouse IgG2b; Invitrogen) and cleaved Caspase3 protein (Anti-ACTIVE Caspase-3 pAb; Promega), antibodies were diluted (1:300) in TBT (Tris-buffered saline with 0.1% Triton X-100) with 10% goat serum and then detected using goat anti-mouse IgG conjugated to horseradish peroxidase and developed as described elsewhere (York et al., 2017). For double labeling of *Snail* and Hu, *in situ* hybridization for *Snail* was followed by immediate washes in PBST, and then

immunohistochemistry for Hu. Sectioning (20 μm) was performed on a Vibratome with embryos embedded in 5% agarose.

2.3. CRISPR/cas9-targeting of lamprey snail

To disrupt *Snail* function *in vivo* guide RNAs (gRNAs) targeting the *Snail* genomic coding sequence (*Snail* gRNA1: 5′-CCCCGCACCTTGTGCACTGGACC-3′; *Snail* gRNA2: 5′-CCTGGCGAGGCACGGGCGATG-3′; protospacer adjacent motif (PAM) sequences are underlined) were delivered by microinjection (described below). We used several different, but complementary, methods when selecting gRNAs. First, we used CRISPOR software to computationally identify optimal gRNA target sites within the *Snail* genomic coding sequence obtained from the recently completed sea lamprey germline genome assembly (http://crispor.tefor.net/) (Concordet and Haeussler, 2018; Smith et al., 2018). We then supplemented our computational approach by taking into account features that have been optimized for gRNA selection in lamprey (Square et al., 2015; York et al., 2017, 2018): 50–80% GC content, with targeted regions as close as possible to the presumptive start codon (or 5′ end of available genomic sequence). All gRNAs were then prepared according to a previously published protocol (Square et al., 2015). Approximately 1hr after fertilization, zygotes were injected (~5 nl) with a cocktail containing 1 ng-ul^{-1} Cas9 protein (PNA Bio), 500 pg gRNA and 10% fluorescein dextran tracer, prepared in nuclease-free water. After waiting 10 min for the Cas9-gRNA complex to form, approximately 5000 embryos were microinjected for several hours, with replicates of these injections performed multiple times over the summer breeding season. Injected embryos were screened by fluorescence three or four days later and non-fluorescent embryos were discarded. Successfully injected embryos were raised to appropriate stages, fixed in MEMFA, and then dehydrated and stored at −20 °C in 100% methanol until needed for analysis.

2.4. Control CRISPR experiments

To control for potential toxicity of Cas9 protein and gRNAs, as well as other unforeseen effects resulting from microinjection, we microinjected the same concentration of Cas9 protein (1 ng-ul^{-1}) and a "scrambled" negative control gRNA (500 pg; 5′-AATAAGTTGGGGTTTCCA-3′) into zygotes from each cohort of fertilized eggs that were microinjected with our *Snail* gRNAs. All control embryos analyzed had the same morphological appearance and gene expression patterns as un-injected wildtype embryos.

2.5. Genotyping of individual CRISPR mutant embryos

Following immunostaining or *in situ* hybridization, we selected five embryos to link individual gene expression phenotypes to a specific mutant genotype. To control for the possibility that tissue fixation and/or damage to genomic DNA during the *in situ* hybridization or immunostaining protocols did not generate erroneous "mutations" during sequencing, the sequences of putative mutant embryos were compared to negative control embryos (see above) that were fixed and assayed by *in situ* hybridization or immunostaining (see also York et al., 2018). These embryos were incubated 24–48 h with 0.1 mg ml^{-1} proteinase K; genomic DNA was extracted using standard methods (Sive et al., 2000). Oligonucleotides (Sigma) surrounding the *Snail* (forward: 5′-GACGGAGCAGCAGAACGATGGT-3′; reverse: 5′-ACCGCTCCCCATAAAACACGC-3′) CRISPR target site were used to PCR amplify and sequence the locus. For each embryo, four different clones were sequenced to document mutagenesis. Our results confirmed that these embryos were actual mutants, thereby effectively linking *Snail* CRISPR phenotypes with specific mosaic mutant genotypes (Fig. S4, 19/20 mutant alleles, 95% efficiency).

2.6. Estimating efficiency of CRISPR/Cas9 mutagenesis

The efficiency of mutagenesis at the *Snail* genomic locus was estimated by pooling 5 randomly selected *Snail* gRNA1 CRISPR-injected embryos at T26. Genomic DNA was isolated per standard methods, the targeted locus was PCR amplified (oligonucleotide sequences listed above), and 40 clones were sequenced. Efficiency (%) was calculated by dividing the number of mutant genotypes by the total number of clones sequenced (see Fig. S5). Injections targeting *Snail* proved to be highly efficient at inducing mutations in these randomly selected embryos, with an estimated mutagenesis efficiency of 90% (36/40 mutant alleles), and 73% (29/40) of these being out-of-frame (Fig. S5).

2.7. Evaluation of off-target CRISPR sites

As described above, our *Snail* gRNAs were designed to minimize potential mutagenesis at off-target loci. Nevertheless, we verified that *Snail* gRNA1 mutant phenotypes were specific to cleavage at the *Snail* locus. To do this, we conducted a BLAST search targeting sequences most similar to the *Snail* gRNA1 sequence in the sea lamprey genome (https://genome.ucsc.edu/cgi-bin/hgGateway). This search revealed that the top three potential off-target genomic loci that contained a PAM cleavage sequence (NGG or reverse complement, CCN) had numerous mismatches, with several of these occurring in the 13 bp "seed sequence" proximal to the PAM site (see Table S2). Two or more mismatches within the seed sequence are often sufficient to prevent off-target mutagenesis (Hsu et al., 2013; Pattanayak et al., 2013). Hence, these potential off-targets are unlikely to be cleaved. To confirm this, genomic DNA isolated from the same five pooled *Snail* CRISPR-injected embryos (Tahara stage 26) that were used to calculate mutagenesis efficiency (see above) was also used to PCR amplify and sequence 10 clones that encompassed these potential off-target regions (see Table S2, Fig. S6) using the following primers: blood plasma apolipoprotein LAL2, forward: 5′-CTTCAGGCCAGTCACCAATG-3′, reverse: 5′-GATGAGGCTTCGATCCATCA-3′; CD45, forward: 5′-TATCACGATCCCTTCAGCTC-3′, reverse: 5′- CACTCAACATAAGCCTGCCA-3′; variable lymphocyte receptor B, forward: 5′-TCGAGAGGCTGCATAGCTAC-3′, reverse: 5′-GTCATGGCAAGCCGTGCGTT-3′. Sequencing revealed no evidence of mutations at these potential off-target sites, which suggests that embryonic CRISPR phenotypes are specific to mutagenesis at the targeted *Snail* locus (Fig. S6).

2.8. Measures of spatial gene expression in neural plate border, preplacodal ectoderm and neural plate

To test if *Snail* regulates medial-lateral gene expression patterning in the neuroectoderm, we quantitatively compared spatial expression patterns of genes that maintained expression in the neural plate border (*Pax3/7*, *Tfap2a*) and neural plate (*SoxB1a*) in *Snail* CRISPR mutants (see Results). To do this, we measured the total width (μm) of the dorsal surface of T17 embryos and then measured (μm) either the total width of expression (for *Pax3/7* and *SoxB1a*) or the width of the non-expressing area between the neural plate borders (for *Tfap2a*) for controls and *Snail* CRISPR mutants. We standardized these values across individual embryos by creating an index of spatial gene expression that divided expression width by embryonic width. Data were non-normally distributed for measures of *Pax3/7* and *SoxB1a* expression (Shapiro-Wilks, p's < 0.01), so we compared indices using a Wilcoxon Rank Sum test. Because measures for *Tfap2a* were normally distributed (Shapiro-Wilks, $p = 0.91$), we used a *t*-test. All analyses were performed in R (R Development Core Team, 2013). Graphical representation of indices as a box and whisker dot plot was performed in R using the package 'ggplot2' (Wickham, 2016). Measurements are in Supplementary Data File 1.

2.9. Character state reconstruction of snail expression domains

Character state reconstruction analysis for categorical data was

performed in Mesquite (Maddison, 2008). Expression patterns of eumetazoan *Snail* homologs in the CNS/PNS, placodes, neural crest, and mesoderm were obtained from the literature and organized into a character matrix (character state present = 1; absent = 0). Default parameters were chosen for the "parsimony ancestral states" analysis option. The data matrix and supporting references are in Table S1.

3. Results

3.1. Molecular phylogenetics of vertebrate snail genes

As a first step toward exploring a possible developmental link in *Snail* activity among neural crest, placodes, and CNS/PNS neurons in lamprey embryos, we characterized the genomic complement of the *Snail* family in the lamprey genome. In jawed vertebrates, *Snail* family genes include *Snail1*, *Snail2* and *Scratch*, with some of these having undergone independent duplications in certain lineages (e.g., teleosts, Thisse et al., 1995; Thisse et al., 1993). Our previous work, coupled with BLAST searches, library screenings, and phylogenetic analysis of *Snail* genes from the sea lamprey germline and somatic genomes consistently reveal a single lamprey *Snail* orthologue residing at the base of the vertebrate *Snail1/Snail2* clade (Fig. S1), as well as a putative *Scratch* orthologue nested within the *Scratch* clade (Rahimi et al., 2009; Smith et al., 2013, 2018; York et al., 2017). Although lampreys may have lost a second *Snail* gene copy, searches of the published *P. marinus* somatic and germline genomes (Smith et al., 2013, 2018), coupled with our phylogenetic analysis, nonetheless support the notion that lampreys contain a single *Snail* orthologue, a feature also shared with hagfish, the sister group to lampreys (Ota et al., 2007).

3.2. Snail regulates neural plate border and pre-placodal ectoderm, but not neural plate

Previous studies have documented expression of *Snail* in early lamprey embryos, with transcripts localizing contiguously in the neural plate, neural plate border, and pre-placodal ectoderm, and maintenance of overlapping CNS-neural crest expression in the neural tube (Rahimi et al., 2009; Sauka-Spengler et al., 2007; York et al., 2017; see also Fig. S2). Based on these overlapping expression patterns, we asked if there might be a *Snail*-mediated functional link among these embryonic territories. To test this, we used CRISPR/Cas9-mediated genome editing to impair *Snail* function and then examined for developmental defects in each cell population.

We previously demonstrated a role for *Snail* during lamprey neural crest migration and differentiation (York et al., 2017). Here, we asked if *Snail* might also be necessary for establishment of the neural plate border GRN module by examining expression of several neural plate border transcription factors, including *Pax3/7*, *nMyc, ZicA*, and *Tfap2a* (Betancur et al., 2010; Milet and Monsoro-Burq, 2012; Nikitina et al., 2008; Plouhinec et al., 2014; Sauka-Spengler et al., 2007). Our results suggested that *Snail* is not required for establishment of the neural plate border via *Pax3/7* (0/12 loss of expression, 0%, Fig. 1A, G). However, we did find that *Snail* patterns the medial-lateral axis of the neural plate border by repressing lateral expansion of *Pax3/7*. Indeed, *Pax3/7* expression expanded laterally by 34% in *Snail* mutants relative to controls ($z_{2,22} = -3.64$, $p = 0.0003$; Fig. 1A, G, M). Our CRISPR knockouts also revealed that *Snail* is required for activation of *nMyc* and *ZicA* in the neural plate border, as evidenced by nearly complete loss of expression of these markers in the embryos analyzed (*nMyc*: 17/18, 94%, Fig. 1B, H; *ZicA*: 14/17, 82%; Fig. 1C, I). By contrast, *Snail* was not required for *Tfap2a* expression (0/11 loss of expression, 0%, Fig. 1D, J), nor for patterning the spatial boundaries of *Tfap2a* expression along the medial-lateral axis of the neural plate border ($t_{2,20} = -0.61$, $p = 0.55$, Fig. 1M).

After demonstrating that *Snail* regulates development of the neural plate border, we next asked if *Snail* might be required for development of the pre-placodal ectoderm, given that its expression extends into this area (Fig. S2). In vertebrates, the pre-placodal domain is delineated in the neuroectoderm just anterior and lateral to the neural plate border, which can be marked in part by expression of *Dlx* cognates in gnathostomes and lamprey (*DlxB*) (Betancur et al., 2010; Sauka-Spengler et al., 2007). Our findings suggest that *Snail* helps establish the pre-placodal ectoderm in lamprey as most embryos (23/26, 88%, Fig. 1E, K) showed a near-total loss of *DlxB* expression anteriorly.

Finally, we tested if *Snail* was required for development of the neural plate by examining expression of *SoxB1a*, a lamprey member of the SoxB family of transcription factors that are important regulators of early CNS development across bilaterians (Pevny and Placzek, 2005; Royo et al., 2011). Although *Snail* has a strong mRNA signal throughout the lamprey neural plate (Fig. S2), we found no evidence that *Snail* is functionally required for *SoxB1a*-mediated establishment of this embryonic domain (0/13 loss of expression, 0%, Fig. 1F, L). In jawed vertebrates, loss of neural plate border transcription factors (e.g., *Snail*) can result in compensatory lateral expansion of neural plate markers (e.g., *SoxB*) into the neural plate border (Langer et al., 2008). However, spatial measures of *SoxB1a* gene expression did not reveal significant differences between controls and *Snail* CRISPR mutants ($z_{2,24} = -0.52$, $p = 0.60$, Fig. 1M). In summary, we found that lamprey *Snail* is essential for proper

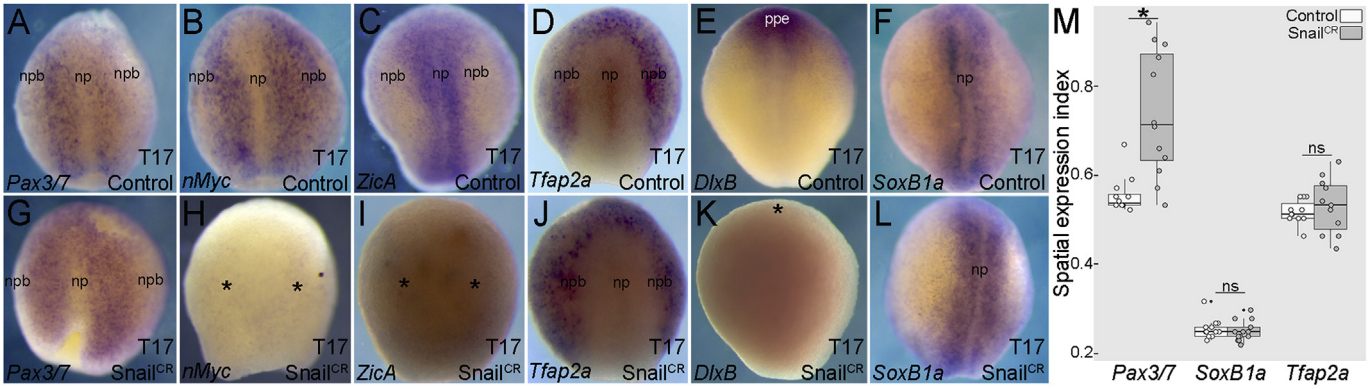

Fig. 1. *Snail* patterns the neural plate border and establishes the pre-placodal ectoderm, but is not required for formation of the neural plate. (A–F) Control T17 embryos with expression patterns delineating the neural plate border (npb, *Pax3/7*, *nMyc, ZicA, Tfap2a*), pre-placodal ectoderm (ppe, *DlxB*), and neural plate (np, *SoxB1a*). (G–L) T17 *Snail* CRISPR mutants (Snail^{CR}) show lateral expansion of *Pax3/7* expression (compare expression between np and npb in A and G), and loss of *nMyc* and *ZicA* expression in the np and npb (asterisks, H, I). (J) *Tfap2a* expression in the nbp appears normal in Snail^{CR} embryos. (K) Loss of *DlxB* transcripts in ppe (asterisk) is observed in Snail^{CR} embryos, whereas no obvious change in *SoxB1a* expression is observed in the np (L). (M) Quantitative comparisons of spatial gene expression indices in controls versus Snail^{CR} embryos (see Materials and Methods for details). Asterisk in panel "M" indicates statistically significant difference ($\alpha = 0.05$), whereas "ns" indicates not significant.

development of the neural plate border and pre-placodal ectoderm but is not required for establishment or patterning of the neural plate.

3.3. *Snail* expression prefigures domains of CNS neurogenesis and differentiation

After examining the expression and function of *Snail* in the lamprey neuroectoderm (Fig. 1, Fig. S2), we characterized its expression from the onset of neurogenesis (~T24) into later stages of neural differentiation (~T26) in the CNS and PNS. Following early expression in the neural plate and subsequently closed cranial neural tube (Fig. S2), lamprey *Snail* expression is maintained in the cranial CNS at T24 (Fig. 2A), accumulating in the centrally located ventricular zone (Fig. 2B), where vertebrate CNS neural stem cells arise, and also peripherally in the marginal zone (Fig. 2B) where some of the earliest differentiated neurons first appear, as evidenced by immunostaining for the neuron-specific differentiation marker, Hu (Fig. 2C and D; Temple, 2001). Indeed, at T24 double labeling for *Snail* mRNA and Hu protein revealed partially overlapping expression within the marginal zone (Fig. 2E). At later stages (T26) when *Snail* expression occupies the entire trunk CNS (Fig. 2F, G), a large area of the trunk CNS marginal zone contains Hu-positive neurons (Fig. 2H and I), and this domain overlaps with *Snail* mRNA localization (Fig. 2J). Taken together, these expression patterns demonstrate maintenance of robust expression of *Snail* throughout stages of neurogenesis into neuronal differentiation.

3.4. *Snail* is essential for early stages of PNS, but not CNS, neurogenesis

Based on *Snail* expression from the onset of neurogenesis through neural differentiation (Fig. 2), we asked if *Snail* activity might be essential for each of these processes in the CNS and PNS. We first tested *Snail* function during early neurogenesis in the CNS, which can be tracked by expression of *SoxB1a* at T24/T25 (Uy et al., 2012). Similar to that in the early neuroectoderm, we also found that *Snail* does not seem to be required for the onset of *SoxB1a*-mediated neurogenic expression in the lamprey CNS, during either early (0/22 loss of expression, 0%, T24, Fig. 3A and B) or relatively later (0/16 loss of expression, 0%, T25, Fig. 3C and D) stages of development. We then tested if *Snail* is required for neurogenesis in the cranial PNS by analyzing expression of *Six1/2*, *Pax3/7*, and *Phox2* in neurons of different cranial sensory ganglia at T25 (McCauley and Bronner-Fraser, 2002; Modrell et al., 2014; York et al., 2018). Our functional results suggested that *Snail* activity is essential for the onset of gene expression patterns that promote PNS neurogenesis, as demonstrated by complete or nearly complete loss of expression of *Six1/2* in epibranchial and posterior lateral line ganglia (9/13, 69%), *Pax3/7* in the ophthalmic division of the trigeminal nerve (7/10, 70%), and in *Phox2*-positive epibranchial ganglia (12/17, 71%) (Fig. 3E–J). Moreover, in several of these affected T25 embryos (20/28, 68%) we observed variable levels of abnormal head development, including an overall decrease in head size as well as abnormalities in the oropharynx (e.g., Fig. 3F, H, J; see also Fig. 4H). These variable patterns are most likely linked to variation in loss or reduction of cranial neural crest cells (York et al., 2017), a feature that probably stems from generating mosaic CRISPR/Cas9 mutants (Square et al., 2015; Zu et al., 2016).

3.5. *Snail* is required for the differentiation of CNS neurons

Because our results thus far suggested that *Snail* is not required for establishment of the neural plate at T17 (Fig. 1) or CNS neurogenesis at T24/25 (Fig. 3), we asked if *Snail* was required for neuronal differentiation. We showed recently that two markers of neural differentiation, Hu protein and *Neural Cell Adhesion Molecule* (*NCAM*) mRNA, are expressed in the developing lamprey CNS and PNS (York et al., 2017). Here, we show that knockout of *Snail* function resulted in a complete or nearly complete loss of expression of both Hu ($n = 13/16$, 81%, Fig. 4A–F) and *NCAM* ($n = 16/16$, 100%, Fig. 4G and H) throughout much of the lamprey cranial and trunk CNS. Moreover, consistent with our analysis of *Snail* involvement in PNS neurogenesis (Fig. 3), we also observed loss of Hu ($n = 14/15$, 93%) and *NCAM* ($n = 15/15$, 100%) expression in PNS sensory neurons in the head and trunk (Fig. 4A–H).

3.6. *Snail* regulates CNS differentiation by promoting cell survival

Snail has been shown to regulate apoptosis and cell proliferation (Metzstein and Horvitz, 1999; Thellmann et al., 2003; Vega et al., 2004). Thus, we tested if increased cell death and/or decreased cell proliferation might account for the apparent loss of CNS neural marker expression in *Snail* CRISPR mutants by examining CNS expression of Caspase3 protein and *Proliferating Cellular Nuclear Antigen* (*PCNA*) mRNA, respectively (Barrallo-Gimeno and Nieto, 2005; Campbell et al., 2018; Lara-Ramirez et al., 2019; Metzstein and Horvitz, 1999; Vega et al., 2004). *Snail* CRISPR mutants had extensive apoptosis in the CNS ($n = 5/8$, 63%, Fig. 4I, K), with most apoptotic cells occurring in the marginal zone where neurons are undergoing differentiation. By contrast, there appeared to be no appreciable change in *PCNA* expression in the neural stem cell-producing ventricular zone within the CNS of *Snail* mutants ($n = 0/8$, 0%, Fig. 4J, L).

3.7. Ancestral state reconstruction reveals an ancient snail-positive domain in the bilaterian CNS and CNS-neural crest-placode function in early vertebrates

Our combined expression and functional analyses point to an important role for lamprey *Snail* in promoting the development of CNS

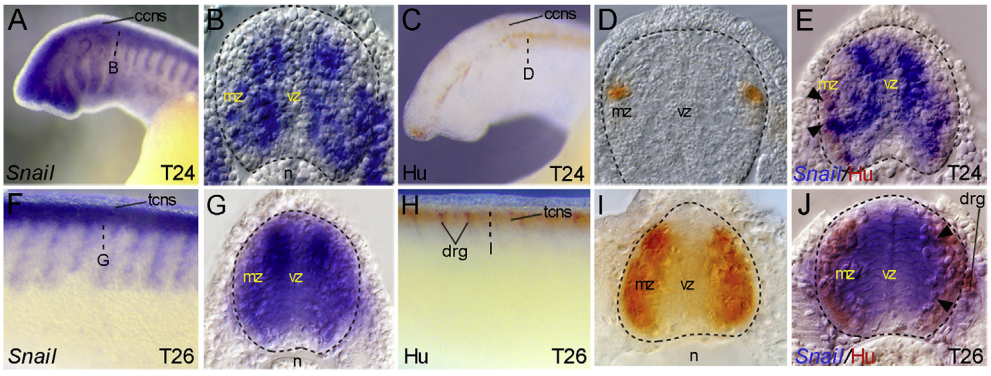

Fig. 2. *Snail* is expressed in the cranial and trunk CNS throughout neurogenesis. (A,B) During early CNS neurogenesis (T24), *Snail* is enriched in the cranial CNS (ccns) ventricular zone (vz), and especially the marginal zone (mz; neural tube outlined, B), where Hu expression in ccns mz neurons appears (C; neural tube outlined in D). (E) *Snail* and Hu expression at T24 overlaps in neural tube mz (arrowheads, neural tube outlined). In older embryos (T26), *Snail* mRNA in the trunk CNS (tcns in F) includes the vz and mz (G, neural tube outlined). At T26, differentiated tcns neurons and neural crest-derived dorsal root ganglia (drg) express Hu (H), with tcns Hu expression mostly in the mz (I, neural tube outlined). *Snail* and Hu expression at T26 overlaps in the mz (arrowheads in J, neural tube outlined), and weakly in drg (drg in J). Other abbreviations: n, notochord.

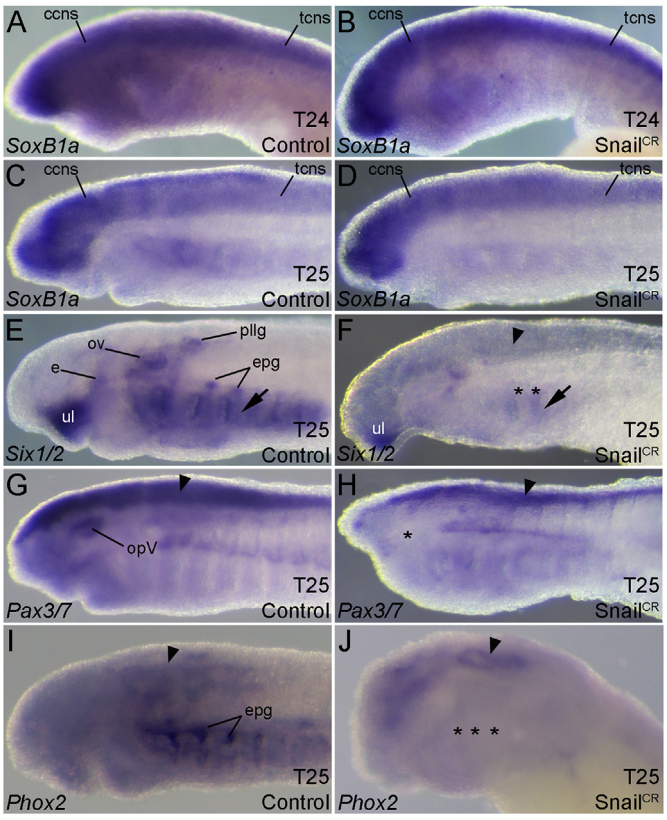

Fig. 3. *Snail* is not required for early CNS neurogenesis but is essential for formation of cranial PNS neurons. (A) T24 control embryo showing *SoxB1a* neurogenic expression in the cranial and trunk CNS (ccns, tcns). (B) *Snail* CRISPR mutant (Snail^{CR}) shows no obvious loss of *SoxB1a* expression in ccns or tcns. (C) *SoxB1a* expression at T25 showing continued neurogenic expression of *SoxB1a* in ccns and tcns with (D) no appreciable change in expression in Snail^{CR} embryos. (E) *Six1/2* expression in neurons of the cranial PNS (pllg, epg), upper lip (ul) and pharyngeal arches (arrow) of control embryo. (F) Snail^{CR} embryos show nearly complete loss of cranial neural expression in the PNS (asterisks, loss of epg; arrowhead, loss of pllg), and expression in pharyngeal arches (arrow). (G) Control embryo with *Pax3/7* expression in the CNS (arrowhead) and placode-derived portion of opV neurons in the PNS. (H) Mutant embryo showing abrogated *Pax3/7* neuronal expression in the opV (asterisk) but retention of CNS expression (arrowhead). (I) Control embryo with expression of *Phox2* in the CNS (arrowhead) and in PNS neurons in epg. (J) Snail^{CR} embryos show complete loss of *Phox2* expression in epg (asterisks), but maintain CNS expression (arrowhead). Abbreviations: e, eye; epg, epibranchial ganglion; opV, ophthalmic division of the trigeminal ganglion; ov, otic vesicle; pllg, posterior lateral line ganglion.

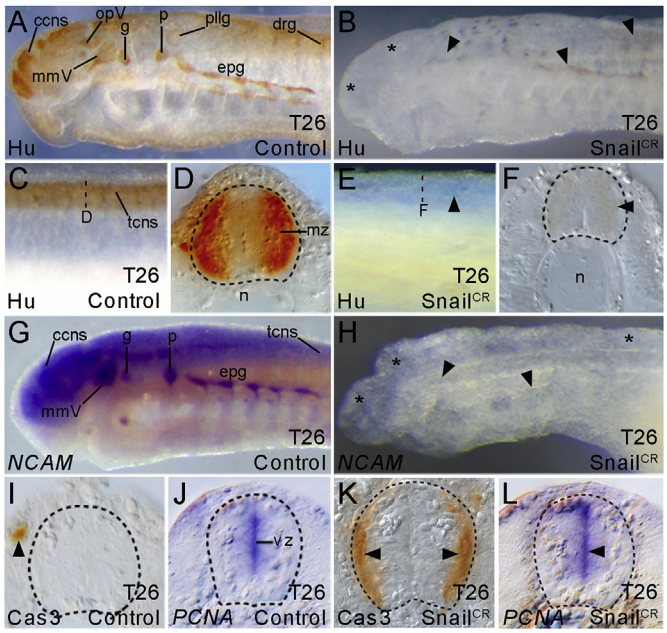

Fig. 4. *Snail* is required for CNS neural differentiation by enhancing cell survival. (A) T26 control embryo expressing the neural differentiation marker Hu in cranial CNS (ccns) neurons, cranial PNS neurons (opV, g, p, pllg, epg), and neurons of trunk dorsal root ganglia (drg). *Snail* CRISPR (Snail^{CR}) mutants lose Hu in the ccns (asterisks, B) and cranial PNS neurons (arrowheads, B). (C) Hu expression in the trunk CNS (tcns) at T26 is prominent in the marginal zone (D, mz, neural tube outlined). Mutant embryos show loss of Hu-positive neurons in the tcns (arrowhead, E and arrowhead, F, with neural tube outlined). (G) Control *NCAM* expression at T26 in ccns, tcns and cranial PNS neurons. Snail^{CR} embryos lose *NCAM* expression in the ccns and tcns (asterisks in H), and in cranial PNS neurons (arrowheads, H). (I) Control T26 neural tube cross section showing no cell death in the tcns (neural tube outlined, arrowhead shows dying cell outside neural tube). (J) Control T26 neural tube cross section in the tcns showing normal *PCNA* expression in the ventricular zone (vz). (K, L) Snail^{CR} embryos show increased apoptosis in the periphery of the tcns (arrowheads, K, neural tube outlined), but no appreciable change in *PCNA* expression (arrowhead, L, neural tube outlined). Abbreviations: epg, epibranchial ganglion; g, geniculate ganglion; mmV, maxillomandibular part of the trigeminal ganglion; n, notochord; opV, ophthalmic part of the trigeminal ganglion; p, petrosal ganglion; pllg, posterior lateral line ganglion.

neurons, in addition to roles in neural crest and placode development at various stages (Figs. 1–4). Interestingly, these multi-functional roles in lamprey share similarities with vertebrates on one hand (*Snail* in neural crest, placodes) and invertebrates on the other (*Snail* in CNS/PNS neurogenesis). We asked if these expression domains reflect phylogenetically conserved states by mapping *Snail* expression patterns (CNS/PNS neurons, neural crest, placodes, mesoderm) onto a consensus eumetazoan phylogeny using character state reconstruction analysis (Fig. 5, Fig. S3, Table S1). We found that expression in the mesoderm (or endoderm in diploblasts) probably represents the most ancient expression domain of *Snail* (Fig. S3). We also found support for an ancestral *Snail* expression domain in CNS/PNS neurons that likely dates back to the last common ancestor of bilaterians (Fig. 5). Finally, our analysis suggested that a *Snail*-positive CNS-"proto-neural crest"-"proto-placode" domain was likely present in the last common ancestor of tunicates and vertebrates, with *Snail* likely regulating an overlapping CNS-neural crest-placode domain in ancestral vertebrates, but being lost in one or more of these domains in some taxa (Fig. 5).

4. Discussion

Neural crest cells and placodes are hallmarks of the vertebrate clade (Green et al., 2015; Meulemans and Bronner-Fraser, 2005). Yet, the mechanisms underlying the integration of key genes, such as *Snail*, into neural crest and placode regulatory networks from their pre-vertebrate origins have remained elusive. Our results here highlight key points of conservation, but also divergence, in the deployment of *Snail* not only between lamprey and jawed vertebrates, but also across a diverse range of eumetazoan taxa, with implications for the origin of neural crest and placode regulatory mechanisms.

4.1. An ancient role for snail in patterning bilaterian neuroectoderm and regulating neurogenesis

One of the surprising features of lamprey embryogenesis that we have described here is the persistent expression of *Snail* in neurogenic tissues, particularly in the CNS. *Snail* is expressed in lamprey throughout the neural plate and then maintains robust expression within the neural tube from neurogenesis to terminal differentiation of neurons (Fig. S2, Fig. 2). Expression of *Snail* in the lamprey neural tube was noted briefly in an

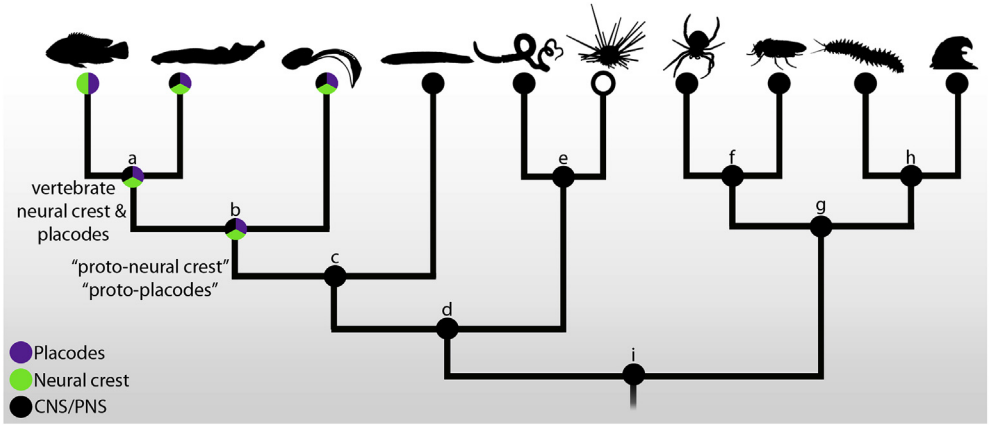

Fig. 5. Model for the evolution of *Snail*-mediated regulation of neural crest and placodes from an ancient role in bilaterian neurogenesis. Terminal branches, from left to right, show representative bilaterian groups including bony fish (jawed vertebrate), lamprey (jawless vertebrate), tunicate (invertebrate chordate), amphioxus (invertebrate chordate), acorn worm (hemichordate), sea urchin (echinoderm), spider (arachnid), fly (insect), polychaete worm (annelid), snail (mollusc). Common ancestors, indicated by lower case letters at node points, include "a", vertebrates; "b", olfactores; "c", chordates; "d", deuterostomes; "e", ambulacraria; "f", ecdysozoa; "g", protostomia; "h", lophotrochozoa; "i", bilateria. Circles with color schemes at terminal branches and ancestral nodes denote *Snail* expression domains in placodes (purple), neural crest (green) and/or CNS/PNS neurons. Ancestral state reconstruction places *Snail* expression in the CNS/PNS/neuroectoderm as ancestral for bilaterians (black circle at "i", 2 character changes required), with secondary loss (empty circle) of this domain in invertebrate deuterostomes such as echinoderms. In the olfactorean ancestor, *Snail* acquired expression in "proto-neural crest" and "proto-placodes" and patterned the neural plate and CNS. At the origin of vertebrates, evolutionarily conserved *Snail* CNS expression was retained and acquired downstream transcriptional targets such as *Pax3/7*, *SoxE*, *nMyc* and type II cadherins during the evolution of bona fide neural crest (see Results and Discussion). This ancestral CNS-neural crest-placode function for *Snail* is retained in lamprey.

earlier study (Rahimi et al., 2009), but the authors did not investigate the significance of this observation. This particular pattern has not, to our knowledge, been described in any jawed vertebrate, but there are striking parallels among several invertebrates. For example, a lamprey-like pattern of *Snail* CNS expression occurs in the invertebrate chordates, amphioxus and *Ciona* (Hudson et al., 2015, 2018; Langeland et al., 1998). Similarly, *Snail* is expressed throughout the neuroectoderm of invertebrate deuterostomes such as hemichordates (Green et al., 2013). These patterns can also be found in diverse lophotrochozoans and ecdysozoans, including annelids, molluscs, insects, arachnids, crustaceans, and nematodes where *Snail*-positive cells occur in neural precursors and/or ventral nerve cord (Ashraf and Ip, 2001; Dill et al., 2007; Kerner et al., 2009; Kim et al., 2017; Klann and Stollewerk, 2017; Lespinet et al., 2002; Metzstein and Horvitz, 1999; Nieto, 2002; Sommer and Tautz, 1994; Southall and Brand, 2009; Ungerer et al., 2011; Weller and Tautz, 2003). Based on these shared patterns of expression and ancestral state analysis (Fig. 5), we propose that *Snail* was deployed in ancestral bilaterians for regulating CNS and neural development, in addition to mesoderm specification (Fig. S3).

Although we find overall patterns of evolutionary conservation between lamprey and invertebrates for a CNS-neurogenic role for *Snail sensu lato*, there are also lineage-specific differences, particularly in the extent and timing of expression. For example, expression of *Snail* in lamprey is ubiquitous in the neuroectoderm with gradual resolution to more discrete domains within neurogenic tissues (e.g., CNS marginal zone, Fig. S2, Fig. 2). By contrast, *Snail* expression in invertebrate chordates such as amphioxus similarly begins as broadly neuroectodermal, but then quickly sharpens to the neural plate border, only to be upregulated secondarily throughout the larval CNS in a pattern even broader than that of lamprey (Langeland et al., 1998). In tunicates, *Snail* expression in the early gastrula patterns the neural plate and fates specific cells to the lateral CNS and is then upregulated in the cerebral vesicle of the tadpole larva (Esposito et al., 2017; Hudson et al., 2015, 2018; Imai et al., 2006, 2009). There is also variation in *Snail* neurogenic expression among lophotrochozoans and ecdysozoans. *Snail* expression occurs in neural precursors and sensory organs in insects, arachnids and crustaceans, but also in differentiating neurons in some of these clades (Dill et al., 2007; Kerner et al., 2009; Lespinet et al., 2002; Weller and Tautz, 2003). In annelids and molluscs, there are typically two *Snail* paralogs, with expression in the ventral nerve cord and in cells fated to become paired ventral ganglia (Dill et al., 2007; Kim et al., 2017; Lespinet et al., 2002; Osborne et al., 2018).

In contrast to these examples of conservation, not all bilaterians have retained *Snail* for a neurogenic capacity. In echinoderms, *Snail* is expressed in mesodermal cells, particularly those that are migrating or ingressing, but there is no evidence that *Snail* is ever expressed during neurogenic patterning or differentiation (Saunders and McClay, 2014; Wu and McClay, 2007). This may be related to the radical reorganization of the bilaterally symmetrical CNS in ancestral echinoderms to one of pentameral symmetry that characterizes extant clades (Dominguez et al., 2002; Gee, 1996, 2018). This dramatic morphological alteration to the echinoderm CNS appears to have been accompanied by commensurate

changes in gene expression patterns, as indicated by, for example, the lack of expression of *Hox* genes in the embryonic nervous system (Arenas-Mena et al., 2000; Gee, 2018). Similarly, in hagfish *Snail* does not appear to be expressed in the CNS, presumably reflecting secondary loss of expression after splitting from the lamprey lineage (Ota et al., 2007), although there is yet to be a comprehensive analysis of *Snail* expression in this cyclostome group. In addition to spatiotemporal variation in expression, there is also evidence for functional variation in *Snail* regulatory activity. In insects, *Snail* regulates neurogenesis by controlling asymmetric cell divisions of daughter cells through *Prospero*, whereas in other invertebrates, *Snail* promotes neuroblast fate and survival (Ashraf and Ip, 2001; Lai et al., 2012; Metzstein and Horvitz, 1999; Thellmann et al., 2003; Weller and Tautz, 2003). Our results here in lamprey suggest that *Snail* may be involved in neural differentiation within the CNS by promoting cell survival rather than by controlling early neurogenesis. Regardless of these heterochronic and heterotopic shifts in *Snail* expression, or variation in function, our analysis nonetheless points to a general domain of evolutionarily conserved *Snail* activity in bilaterian nervous systems.

4.2. *Snail patterning of neural crest and placode territories in the vertebrate neuroectoderm*

We have identified important roles for *Snail* in the establishment, maintenance and patterning of the neuroectoderm in the lamprey embryo that highlight important similarities with jawed vertebrates on one hand and invertebrates on the other. Our results suggest that *Snail* plays at least two important roles early in lamprey neural crest development by regulating the neural plate border. First, *Snail* acts as a transcriptional repressor by setting the boundaries to *Pax3/7* expression laterally, thereby ensuring proper patterning of the medial-lateral axis of the neural plate border. In this regard *Snail* function in the lamprey neuroectoderm is similar to that of *Snail* governing medial-lateral patterning in the neuroectoderm of tunicates and points to a recurring role for *Snail* in bilaterians as a transcriptional repressor to enforce embryonic territorial boundaries (Fujiwara et al., 1998; Kosman et al., 1991; Leptin, 1991). Second, we find that *Snail* is required for activation of *nMyc* and *ZicA* expression in the neural plate border, a result which places *Snail* relatively high within the neural plate border and neural crest specifier modules in the lamprey neural crest GRN. Although these findings support a general role for *Snail* in regulating the neural plate border in lamprey, it is worth pointing out that in gnathostomes it is *Pax3* and *Zic1* that synergistically activate *Snail2* expression, whereas our results suggest that a single *Snail* orthologue is required for activation of these neural plate border specifiers in lamprey (Sato et al., 2005). Thus, although there is certainly evolutionary conservation of the neural plate border regulatory module across vertebrates in the broad sense (Sauka-Spengler et al., 2007), our results suggest that some of the regulatory "wiring" for early neural crest development may be quite different in lamprey.

In addition to the neural crest, we also found that *Snail* plays a pivotal role in the development of another key vertebrate innovation, cranial ectodermal placodes. Our results suggest that *Snail* is essential for *DlxB*-mediated establishment of the pre-placodal territory in the anterior neuroectoderm. We therefore interpret the loss of cranial sensory ganglia in lamprey *Snail* mutants described here (Figs. 3 and 4) and previously (York et al., 2017) as resulting primarily from genetic ablation of *Snail* during the earliest stages of placode development, although we cannot rule out that these phenotypes may be related to a requirement of *Snail* in placode differentiation. To our knowledge there is no evidence that *Snail* is essential for establishment of the pre-placodal territory in jawed vertebrates. Although this result in lamprey is quite different from that in other vertebrates, there are again interesting parallels to be found among tunicates. For example, in *Ciona*, the *Snail*-positive lateral neural border gives rise to the evolutionary precursors of vertebrate neural crest and placodes, and recent work suggests that these cell populations share a common evolutionary origin (Abitua et al., 2012, 2015; Horie et al., 2018). If this hypothesis is correct, then there should have been significant regulatory overlap in placode and neural crest development during early vertebrate evolution. In support of this, we find that *Snail* is expressed in the pre-placodal ectoderm and neural plate border in lamprey and is essential for early development of each of these populations simultaneously. We therefore propose that the broad expression of lamprey *Snail* in the early neuroectoderm enables the dual regulation of both neural crest and placodes. Similarities in *Snail* activity among lamprey, jawed vertebrates and invertebrate chordates, supports the idea of a pre-vertebrate regulatory link between neural crest and placodes, coupled in part by *Snail*, that was retained in ancestral vertebrates.

4.3. *Implications for evolution of vertebrate neural crest and placodes*

Although there is no evidence that the invertebrate chordate amphioxus has neural crest or placodes, there is now a strong case to be made that tunicates–the sister group to all vertebrates–do have rudiments of each of these cell populations (Abitua et al., 2012, 2015; Horie et al., 2018; Stolfi et al., 2015). Intriguingly, the "proto-neural crest" and "proto-placodes" in tunicates derive from a *Snail*-positive neural plate border, with additional *Snail* expression in the tunicate neural plate and neural tube that patterns the medial-lateral axis and specifies CNS lineages (Abitua et al., 2012, 2015; Hudson et al., 2015, 2018; Stolfi et al., 2015). These shared functions for *Snail* in the CNS, proto-neural crest, and proto-placodes are reminiscent of *Snail* activity among invertebrates (CNS, PNS neurons) and jawed vertebrates (neural crest, placodes), but there has been no evidence thus far for a *Snail*-mediated regulatory link between these populations that spans the invertebrate-vertebrate divide.

Our results here describing the expression and multiple functional roles of lamprey *Snail* in the CNS, neural crest and placodes now provide evidence for such a link. Conservation of *Snail* expression and function in the lamprey CNS, neural crest and placodes, coupled with analysis of *Snail* expression across bilaterians, suggests a new hypothesis for the integration of *Snail* into the ancestral neural crest regulatory network and for regulation of placodes. In the last common ancestor of vertebrates and tunicates (i.e., olfactores), we hypothesize that *Snail* simultaneously regulated patterning of the neural plate/CNS as well as precursors of neural crest and placode populations (Fig. 5). During the invertebrate chordate-vertebrate transition (Fig. 5), we propose that the ancestral CNS expression domain of *Snail* was retained in jawless vertebrates and acquired novel functions and transcriptional targets (e.g., *SoxE*, *ZicA*, *nMyc*, *Pax3/7*) in bona fide neural crest and placode development, with secondary functions relating to neural crest migration and differentiation (Fig. 5; York et al., 2017). Ancestral vertebrates (Fig. 5) therefore would have had multiple functional roles for *Snail* including a symplesiomorphic function in CNS neurogenesis, and apomorphic functions in development of neural crest and placodes, with all of these roles being retained in lamprey (Fig. 5). As a corollary, our model predicts that the lack of a CNS-neural crest-placode function for *Snail* in jawed vertebrates may be the result of loss of *Snail* expression in much of the CNS proper, but retention of *Snail* in the dorsal neural tube for neural crest specification (Fig. 5). Similarly, this predicts that hagfish, too, have lost these ancestral domains (Ota et al., 2007), with trait loss being a common occurrence as a result of their derived life history. Despite these variations, our findings nonetheless identify ancestral jawless vertebrates as occupying a key node intermediate to that of invertebrates (*Snail* in neurogenesis) and higher jawed vertebrates (*Snail* in neural crest), with a multi-functional role for *Snail* in CNS neurogenesis, neural crest and placode development.

We have recently shown that regulators of neural crest epithelial-mesenchymal transition (EMT) and migration in lamprey, including *Sip1*, *Zeb1* and *CadIIA* are co-expressed with *Snail* in a similar pattern throughout the CNS, concomitant with specification and migration of neural crest and CNS neurogenesis (York et al., 2017). These results raise the possibility that, similar to *Snail*, these and possibly other transcriptional regulators of neural crest development may have had their origins

in early vertebrate evolution playing roles in both CNS neurogenesis and neural crest/placode development that eventually became partitioned exclusively to neural crest and/or placodes while losing the ancestral CNS neurogenic function in jawed vertebrates.

Finally, it is important to note that *Snail* is a key regulator of mesoderm development, and thus expression of *Snail* in mesodermal tissues points to an alternative hypothesis in which *Snail*-mediated control of neural crest and placode development could have been co-opted from a pre-existing mesodermal program (Fig. S3). Our results here, however, point to a deeply conserved role for *Snail* in the CNS-PNS-neuroectoderm that predated vertebrates and was then coupled to a novel neural crest and placode function early in olfactorean and vertebrate evolution (Fig. 5). Importantly, there is also evidence that *Snail*, along with *FoxD*, *SoxE*, and other neural crest transcriptional regulators are all co-expressed in the neuroectoderm and PNS of invertebrate deuterostomes and protostomes, a pattern not found in the mesoderm of these same groups (Lauri, 2013). Indeed, evidence from annelids suggests that transcriptional regulators and differentiation markers within the neural crest GRN (e.g., *Prdm1, ColA, Brn3, Msx, Olig*) are all expressed with *Snail* in the larval CNS and/or PNS, rather than mesoderm (Lauri, 2013). Those observations support the notion that *Snail* may have been co-opted for neural crest and placode regulation, possibly from within a larger, conserved transcriptional network operating within CNS and PNS neurons that has deep origins in early bilaterian animals. Genome wide-regulatory analysis comparing neural crest and placode programs with those governing mesodermal and neuroectodermal development across vertebrates and invertebrates will help address this possibility.

Competing interests

No competing interests declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.06.010.

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