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Conserved Hox response elements in vertebrate Mox1 loci

Divergent functions of paralogous cis-elements

Analyses of lamprey *meis* genes reveals that conserved inputs from Hox, Meis and Pbx proteins control their expression in the hindbrain and neural tube

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Running title: *meis* genes in lamprey head development

Abstract:

Meis genes are known to play important roles in the hindbrain and neural crest cells of jawed vertebrates. To explore the roles of *Meis* genes in head development during evolution of vertebrates, we have identified four *meis* genes in the Sea Lamprey genome and characterized their patterns of expression and regulation, with a focus on the hindbrain and pharynx. Each of the lamprey *meis* genes displays temporally and spatially dynamic patterns of expression, some of which are coupled to rhombomeric domains in the developing hindbrain and select pharyngeal arches. Studies of *Meis* loci in mouse and zebrafish have identified enhancers that are bound by Hox and TALE (Meis and Pbx) proteins, implicating these factors in the direct regulation of *Meis* expression. We examined the lamprey *meis* loci and identified a series of *cis*-elements conserved between lamprey and jawed vertebrate *meis* genes. In transgenic reporter assays we demonstrated that these elements act as neural enhancers in lamprey embryos, directing reporter expression in appropriate domains when compared to expression of their associated endogenous *meis* gene. Sequence alignments reveal that these conserved elements are in similar relative positions of the *meis* loci and contain a series of consensus binding motifs for Hox and TALE proteins. This suggests that ancient Hox and TALE-responsive enhancers regulated expression of ancestral vertebrate *meis* genes in segmental domains in the hindbrain and have been retained in the *meis* loci during vertebrate evolution. The presence of conserved Meis, Pbx and Hox binding sites in these lamprey enhancers links Hox and TALE factors to regulation of lamprey *meis* genes in the developing hindbrain, indicating a deep ancestry for these regulatory interactions prior to the divergence of jawed and jawless vertebrates.

1. Introduction

Meis proteins are a highly conserved family of transcription factors that have key roles in many aspects of embryonic and axial patterning across metazoans (Burglin, 1997; Lopez-Delgado et al., 2021). In vertebrates, this includes roles in the gene regulatory networks involved in specifying anterior-posterior (A-P) patterning in the developing hindbrain and cranial neural crest (NC) via the clustered *Hox* genes (Choe et al., 2014; Choe et al., 2009; Choe et al., 2002; Deflorian et al., 2004; Machon et al., 2015; Moens and Selleri, 2006; Stedman et al., 2009). The characterization of *Hox*-responsive *cis*-regulatory elements that are involved in these interactions in both vertebrate and invertebrate species has revealed crucial roles for Pbx/Exd and Meis/Hth as *Hox* cofactors (Chan et al., 1994; Ferretti et al., 2000; Merabet et al., 2007; Popperl et al., 1995; Rieckhof et al., 1997; Ryoo and Mann, 1999). Pbx and Meis are members of the TALE (three amino-acid loop extension) family of transcription factors (Burglin, 1997) and have both independent and general roles as cofactors for a variety of transcription factors, with diverse inputs into cell and developmental processes (Laurent et al., 2008; Lopez-Delgado et al., 2021; Merabet and Mann, 2016; Moens and Selleri, 2006; Schulte and Frank, 2014).

In vertebrates, the developing hindbrain forms a series of seven transient lineage-restricted segmental subdivisions, called rhombomeres (r1-r7), with distinct combinations of *Hox* genes being expressed in each rhombomere (Alexander et al., 2009; Lumsden, 2004; Parker and Krumlauf, 2017). This nested, rhombomere-specific *Hox* expression imparts segmental identity across the hindbrain, generating segmental patterns of neurogenesis and neural circuit formation along the A-P axis (Amoyel et al., 2005; Briscoe and Wilkinson, 2004; Di Bonito et al., 2013; Gonzalez-Quevedo et al., 2010). *Hox* expression in the developing nervous system also influences subsequent craniofacial patterning via neural crest cells (Hunt et al., 1991; Minoux and Rijli, 2010; Parker et al., 2018; Trainor and Krumlauf, 2000), which migrate from the neural tube to populate the pharyngeal arches (PA) (Knecht and Bronner-Fraser, 2002; Le Douarin and Kalcheim, 1999; Meulemans and Bronner-Fraser, 2004). *Hox*-TALE interactions play a key regulatory role in segmental patterning of the vertebrate hindbrain (Parker et al., 2016; Parker and Krumlauf, 2020). For example, perturbation of *meis* or *pbx* in zebrafish leads to a 'ground state' in the hindbrain, in which a *Hox*-negative r1-like neural identity is observed across the entire hindbrain (Waskiewicz et al., 2001; Waskiewicz et al., 2002). More recently, *Meis* genes have also been implicated in *Hox*-dependent cranial NC patterning (Amin et al., 2015; Parker et al., 2019b).

Physical interactions between TALE and Hox factors have been shown to modulate binding specificity at *cis*-regulatory sites (Dard et al., 2018; Hudry et al., 2012; Hudry et al., 2014; Joshi et al., 2007; Mann et al., 2009; Merabet and Mann, 2016; Singh et al., 2020). These interactions include formation of Pbx-Hox and Pbx-Meis heterodimers, as well as ternary complexes, which bind to distinct DNA sequences (Berthelsen et al., 1998; Ferretti et al., 2005; Jaw et al., 2000). Combinations of heterodimeric sites are often found in close proximity within *Hox*-responsive enhancers, and these sites can contribute to both activation and repression of target genes (Alexander et al., 2009; Tumpel et al., 2009). Together, these Hox TALE interactions modulate the binding specificity, selectivity and functional outcomes of Hox proteins (Merabet and Mann, 2016). Recent genomic studies of Meis, Pbx, and Hox protein binding in mammalian systems have begun to investigate the properties of their contributions to developmental processes on a genome-wide scale and confirmed many of the mechanistic insights on cooperativity and DNA binding specificity gained from analyzing individual loci (Amin et al., 2015; De Kumar et al., 2017a; Donaldson et al., 2012; Ladam et al., 2018; Penkov et al., 2013; Singh et al., 2020).

The roles of *Hox* genes in segmental patterning of the hindbrain and NC appear to be widely conserved across jawed vertebrates (Lumsden, 2004; Lumsden and Krumlauf, 1996; Parker et al., 2016; Prince et al., 1998). Recent studies in lamprey, a jawless vertebrate, have revealed transient segmental *Hox* expression in similar domains of the hindbrain (Parker et al., 2014a, 2019a). Jawed vertebrate rhombomeric and NC enhancers can also direct segmental expression in transgenic reporter assays in lamprey, suggesting conservation of upstream regulatory inputs in jawed and jawless vertebrates (Parker et al., 2014a; Parker et al., 2019b). These findings reveal ancestral vertebrate *Hox* gene regulatory networks in hindbrain and neural crest patterning that have been retained across extant vertebrates (Parker et al., 2016; Parker and Krumlauf, 2017). Given that regulatory links between *Meis* and *Hox* genes play important roles during hindbrain and pharyngeal patterning in gnathostomes, we sought to investigate whether such interactions may also shape development of the lamprey head.

Previous studies on conserved non-coding elements (CNEs) in vertebrate genomes identified hindbrain enhancers with putative Pbx-Hox and Meis sites that are shared between lamprey and jawed vertebrates (McEwen et al., 2009; Parker et al., 2011). However, these could not be unambiguously associated with a lamprey *meis* gene, and it was not clear whether they were bound by Hox and TALE factors. Subsequent studies have surveyed genome-wide transcription factor binding of Hox, Meis and PBX factors in mouse, identifying key inputs of these factors into *Meis* enhancers (Amin et al., 2015; De Kumar et al., 2017a; Donaldson et al., 2012; Penkov et

al., 2013), but the conservation of such elements in lamprey was unknown. Here, we have leveraged the latest lamprey genome assembly to integrate these findings, expanding our knowledge of lamprey *meis* genes, their expression and regulation. We identify four lamprey *meis* (*A-D*) genes, each expressed in distinct segmental patterns in the developing nervous system and pharynx. Sequence analyses uncover conservation in regulatory elements between lamprey and jawed vertebrates with remarkable conservation of Hox/TALE binding sites across all lamprey paralogs. This suggests that segmental expression of *meis* genes in the developing hindbrain depend upon regulatory interactions between Hox and TALE proteins which have a deep ancestry prior to the divergence of jawed and jawless vertebrates.

2. Materials and methods

2.1. Phylogenetic analysis of lamprey *meis* proteins

Manual curation of lamprey *meis* gene models was carried out using Apollo in JBrowse (Dunn et al., 2019), with these models subsequently being supported by automated computational analysis (NCBI). The four *meis* genes have the following GenBank accession numbers: *meisA* - XM_032964291.1, *meisB* - XM_032964293.1, *meisC* - XM_032965227.1, *meisD* - XM_032962974.1. Predicted Sea Lamprey Meis protein sequences were aligned against homologs from other chordate species, retrieved from GenBank. Predicted hagfish Meis proteins were retrieved from the Eburgeri_3.2 assembly via Ensembl (Howe et al., 2021). In selecting jawed vertebrate taxa for this analysis, we avoided teleost fish and *Xenopus laevis* as these lineages have undergone additional genome duplication events which can lead to their co-orthologous genes/proteins being more derived than those from non-duplicated lineages. We opted for West Indian coelocanth (*Latimeria chalumnae*) as an early diverging Sarcopterygian representative, as coelocanths have slowly evolving protein-coding genes (Amemiya et al., 2013). Chicken (*Gallus gallus*) was used as a bird representative, but *Meis3* is not present in chicken - it has been suggested that *Meis3* was lost in the bird lineage (Sanchez-Guardado et al., 2011). Urochordates are the sister group of vertebrates so *Ciona intestinalis* Meis was used as an outgroup in our analysis. Protein alignments were performed rather than DNA alignments due to the high coding GC content in lamprey, which can result in artefactual clustering of lamprey genes in DNA trees. Nevertheless, the unique pattern of amino-acid composition in lamprey proteins is a complicating factor that impinges on their phylogenetic analysis and can lead to artefactual clustering of lamprey proteins (Qiu et al., 2011). Evolutionary analyses were conducted in MEGAX (Kumar et al., 2018), using the Maximum Likelihood method with 500 replicates for bootstrap testing. There was a total of 642 positions in the final dataset for the alignment that included hagfish, with 617 positions for the alignment that excluded hagfish.

2.2. Lamprey embryos

Lamprey husbandry and embryo collection was performed as previously described (Nikitina et al., 2009) with embryos being staged according to Tahara (Tahara, 1988), fixed in MEMFA, and dehydrated in 100% ethanol for storage at -20°C. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and

protocols were approved by the Institutional Animal Care and Use Committee of the California Institute of Technology (lamprey, Protocol #1436-17).

2.3. Cloning of cDNA for *in situ* hybridization probes

Lamprey *krox20*, *wnt1* probe sequences are as previously reported (Parker et al., 2014a). In-situ probes for *meis* genes were designed based on predicted gene sequences in the sea lamprey germline genome assembly (kPetmar1) (Smith et al., 2018), with care taken to avoid repetitive elements. We designed in-situ probes to target distinct sequences for each gene. For *meisC* and *meisD*, probes were designed to target exonic regions. For *meisA* and *meisB*, we designed probes against their 3'UTR sequences, since exonic probes would cross-hybridize between transcripts for each gene. Probe sequences were amplified from *P. marinus* genomic DNA or from st18-26 embryonic cDNA by PCR using KOD Hot Start Master Mix (Novagen). PCR products were cloned into the *pCR4-TOPO* vector (Thermo Fisher Scientific) and sequenced. The following PCR primers were used for amplifying probe templates, with probe lengths given:

meisA (599 bp, 3'UTR fragment) F: 5'-TGCCCAAGTAAACACGACG-3'; R: 5'-GCTTCTTCTCAATGAGCCGG-3'.

meisB (544 bp, 3'UTR fragment) F: 5'-CTCCAGCTTCCTCGTACTCC-3'; R: 5'-AGTACGGGGCAATGTCACAG-3'.

meisAB (509 bp, partial exons) F: 5'-TACGATGAGCTGGGCCATTA-3'; R: 5'-ATGTACCGGTGGCAGAAGTT-3'. This primer pair targets regions of *meisA* and *meisB* transcripts that differ by only 1bp across 509bp, generating a probe that will bind to transcripts of either gene.

meisC (573 bp, partial exons) F: 5'-CTTTGAGAAGTGCGAGCTGG-3'; R: 5'-GAAAATGCCGCGCTTCTTCT-3'.

meisD (704 bp, partial exons) F: 5'-AGCAAGACTTTAAAGCGGCG-3'; R: 5'-TCACCAGGTCTATCGGCATC-3'.

2.4. *In situ* hybridization

Digoxigenin-labelled probes were generated by standard methods and purified using the MEGAclean Transcription Clean-up Kit (Ambion). Lamprey wholemount in-situ hybridization was performed as described previously (Nikitina et al., 2009; Sauka-Spengler et al., 2007), with the following amendments to the protocol: methanol-stored embryos were first transferred into ethanol and left overnight prior to rehydration; a treatment of 0.5% acetic anhydride in 0.1M triethanolamine was added after proteinase K digestion. Hybridization was performed at 70°C for each probe. Embryos were cleared either by using a glycerol series followed by imaging in 100% glycerol, or by using a 1:2 ratio of benzyl alcohol:benzyl benzoate followed by mounting in Permount (Fisher Scientific) on microscope slides for imaging.

2.5. Sectioning

After in-situ hybridization, selected embryos were transferred to 30% sucrose in phosphate-buffered saline, embedded in O.C.T compound (VWR), and cryo-sectioned to 10µm-thick sections.

2.6. Sequence alignment

Global sequence alignment of *Meis* genomic loci was performed using Multi-LAGAN (Brudno et al., 2003), with mouse as the baseline sequence and conserved sequences defined by 65% conservation over 40bp. Sequence alignments of *Meis* enhancers (Fig.7B) were performed using AlignX in VectorNTI (Life Technologies).

2.7. Enhancer elements

Putative *cis*-elements to assay for enhancer activity were selected based on sequence conservation in cross-species alignments, with approximately 300-500bp of non-conserved flanking sequence included on each side. The DNA for each element was amplified from genomic DNA using KOD Hot Start Master Mix (Novagen). The following primers were used for amplification. The sequences given in uppercase represent homology to genomic DNA, with adaptor sequences for cloning given in lowercase text.

Mouse Meis1 element 1 (1513bp) F: 5'- agggtaatgagggccAGTTTGTAGGTGGGTTTGGAAC - 3'; R: 5'- gaggatatcgagctcgGCTCTAACCTCTCCCAGTCC -3'.

Mouse Meis1 element 2 (1199bp) F: 5'- agggtaatgagggccAGCAGGCAGAGAAGGAAGATAAC - 3'; R: 5'- gaggatatcgagctcgTGATCACCGTCTTCAATACAG -3'.

Mouse Meis2 element 2 (1008bp) F: 5'- agggtaatgagggccGTTTGGGTGGTGGCAAGATTTC -3';
R: 5'- gaggatatcgagctcgCATCCGCTCTTCCTCTCTCC -3'.

Lamprey meisA/B element 2 (1244bp) F: 5'- agggtaatgagggccTAGCACTCCGGTACCCTCTAG -3';
R: 5'- gaggatatcgagctcgGGCAATGTTCAAGAAAGGAAG -3'.

Lamprey meisC element 2 (1276bp) F: 5'- agggtaatgagggccTTTGCAAGGTCAGTCGTGTG -3';
R: 5'- gaggatatcgagctcgAAGGGTACGTCTGTGGAAGG -3'.

Lamprey meisD element 1 (1522bp) F: 5'-
agggtaatgagggccAGTCACCATTGCCACATTCAAAATAAAAC -3'; R: 5'-
gaggatatcgagctcgCACGGACGTACAGTTTGGAC -3'.

Lamprey meisD element 2 (2520bp) F: 5'- agggtaatgagggccACCCCGCCAATAACAACAAG -3';
R: 5'- gaggatatcgagctcgCCAACCGTGCACAGAATACAC -3'.

2.8. Generation of reporter constructs

The HLC vector was created in a previous study (Parker et al., 2014a). PCR-amplified enhancer elements were purified using the QIAquick PCR Purification Kit (Qiagen) and cloned into HLC by Gibson Assembly using the Gibson Assembly Master Mix (NEB).

2.9. Zebrafish reporter assay

The wild-type Slusarski AB zebrafish line was used for embryo micro-injection experiments using Tol2-mediated transgenesis (Fisher et al., 2006). An injection mix containing 25 ng μl^{-1} reporter plasmid (generated by miniprep), 35 ng μl^{-1} Tol2 transposase messenger RNA, and 0.05% phenol red was micro-injected into one-celled embryos at an injection volume of 3–5 nl. Embryos were screened at 24–30 h post fertilization for fluorescent reporter expression using a Leica M205FA microscope. In assaying reporter constructs by transient transgenesis, for each injected construct the tissue-specific GFP expression domains were noted, along with the number and proportion of screened embryos exhibiting GFP expression in each of those domains. The empty HLC reporter vector (without an enhancer) directs weak mosaic GFP expression in multiple cell types including neurons and muscle cells. This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH and protocols approved by the Institutional Animal Care and Use Committees of the Stowers Institute (RK Zebrafish Protocol: #2019-089).

2.10. Lamprey reporter assay

Lamprey transient transgenesis was performed using *P. marinus* embryos at the one-cell stage with I-SceI meganuclease-mediated transgenesis (Parker et al., 2014a; Parker et al., 2014b). Injection mixes containing 20 ng μl^{-1} reporter plasmid (generated by miniprep), 1x CutSmart buffer (NEB), and 0.5U μl^{-1} I-SceI enzyme (NEB) in water were incubated at 37 °C for 30 min prior to micro-injection at a volume of ~2 nl per embryo. Embryos were screened for fluorescent reporter expression using a Zeiss SteREO Discovery V12 microscope. For each injected construct, the tissue-specific GFP expression domains were noted, along with the number and proportion of screened embryos exhibiting GFP expression in each of those domains. The empty HLC reporter vector (without an enhancer) directs GFP expression in ectoderm, yolk cells, as well as in cells dorsal to the yolk (Parker et al., 2019b). Since transient reporter assays generate mosaic reporter expression patterns, variation in levels and domains of GFP expression are observed between embryos. For imaging we selected embryos with GFP-expressing patterns representative of the expression potential of the reporter construct, as inferred from screening more than 100 injected embryos. GFP-expressing embryos were imaged using a Zeiss SteREO Discovery V12 microscope and a Zeiss AxioCam MRm camera with AxioVision Rel 4.6 software. Images were cropped and altered for brightness using Adobe Photoshop CS5.1.

2.11. Imaging

Images of BABB-cleared embryos were taken using a Zeiss Axiovert 200 microscope with an AxioCam HRc camera and AxioVision Rel 4.8.2 software. Glycerol-cleared embryo images were taken using a Leica MZ APO microscope with a Lumenera Infinity 3 camera and Infinity Analyze software. Sections were imaged using a Zeiss Axiovert 200 microscope with a Lumenera Infinity 3 camera and Micro-Manager 1.4.22 software. Images were cropped and altered for brightness and contrast using Adobe Photoshop CS5.1.

2.12. ChIP-seq data analysis

Data for ChIP-seq from mouse pharyngeal arch tissue (Amin et al., 2015; Donaldson et al., 2012) were downloaded as processed reads from EMBL ArrayExpress (Hoxa2: E-MTAB-711, Meis: E-MTAB-1632, Pbx: E-MTAB-1633). Using rtracklayer 1.46.0, liftOver was used to convert the coordinates from mm9 to mm10, then generate coverage objects and export them as bigWigfiles for both IP and input data for each replicate. Data for ChIP-seq from differentiated

mouse ES-cells were downloaded from NCBI Sequence Read archive (SRA; <http://www.ncbi.nlm.nih.gov/sra/>) under BioProject accession numbers PRJNA341679 and PRJNA335616 and analyzed as previously described (De Kumar et al., 2017a; De Kumar et al., 2017b).

2.12 Data –Availability

Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at [<http://odr.stowers.org/websimr/>]. The lamprey genome assembly (kPetMar1) and associated annotations are available from the Stowers Institute at [<https://simrbase.stowers.org/sealamprey>].

3. Results

3.1. The lamprey *meis* complement

To explore the roles of *meis* genes in neural crest cells and the hindbrain during head development in vertebrate evolution we first set out to examine whether their patterns of gene expression and regulation are conserved between mouse and lamprey. Towards this goal, we have identified four *meis* genes in the Sea Lamprey genome (Smith et al., 2018), which occupy three separate genomic loci (Fig. 1A). One of these loci contains a pair of *meis* genes (*meisA* and *meisB*), which are adjacent to each other and oriented in a head to tail fashion. These tandem *meis* genes are highly similar in coding sequence, indicating that they are derived from a tandem duplication event. To address whether this *meisA/B* tandem duplication is lamprey-specific, we compared the lamprey *meis* annotations to those in the hagfish genome assembly (Eburgeri3.2). Of the three annotated hagfish *meis* genes, two are present in tandem and adjacent to the *c15orf41* gene, as are *meisA/B* in lamprey (Fig. 1B). This suggests that they may be orthologous to *meisA* and *meisB*, and that the tandem duplication event preceded the divergence of lamprey and hagfish. Comparison of neighboring genes also reveals high similarity between the loci containing lamprey *meisD* and the other hagfish *meis* gene ('*meis* 6940'), suggesting that these genes are also orthologous. We were unable to identify an ortholog of *meisC* in hagfish, possibly due to the incomplete nature of the genome assembly.

To gain further insight into the relationships between lamprey *meis* genes and their counterparts from other vertebrates, we performed phylogenetic analyses (Fig. 1C,D). We found strong support for the orthology of lamprey *meisD* and hagfish *meis* 6940. The tree also places lamprey *meisA/B* and their putative hagfish orthologs within a clade, although it does not resolve direct orthology between them, and the bootstrap values for these groupings are low. These groupings could reflect either the derivation of these tandem hagfish *meis* genes from an independent duplication event in hagfish, rapid sequence divergence of these genes in hagfish, or an artifact of incomplete/misannotation of the hagfish genes. Without further investigation and validation of the hagfish genes it is difficult to resolve these alternative interpretations.

Comparing lamprey and gnathostome genes, the tree groups *meisA,B,D* with *Meis1* and *Meis2*, while *meisC* is grouped with *Meis3*, although there is only modest bootstrap support for these groupings. Due to the possibility of the hagfish sequences obscuring the phylogenetic relationships, we constructed a tree that excludes the hagfish sequences (Fig 1D). This tree shows similar groupings to the previous tree, but with higher bootstrap support for certain

nodes. *meisA,B,D* group together, nested within a clade containing *Meis1,2*, while *meisC* is grouped with *Meis3*. At face value, this topology suggests that *meisC* is homologous to *Meis3*, and that *meisA/B* and *meisD* are more closely related to *Meis1* and *Meis2* than to *Meis3*. In addition, the clustering of *meisD* with the tandem *meisA/B* genes suggests that these genes derived from a duplication event that occurred in the agnathan lineage.

As an alternative approach for exploring these phylogenetic relationships, we surveyed the complement of syntenic genes linked to each *Meis* locus across lamprey, hagfish and mouse, to search for patterns of conserved synteny (Fig 1B). While conserved synteny is clearly present between lamprey and hagfish, particularly between *meisD* and *meis 6940* loci, there are no obvious or striking patterns of shared gene retention that unequivocally link any lamprey and mouse loci. Taken together, we were able to identify four *meis* genes in lamprey, with *meisA,B,D* seeming to bear higher homology to jawed vertebrate *Meis1,2*, and *meisC* being tentatively grouped with *Meis3*.

3.2. Embryonic expression of lamprey *meis* genes

We investigated the expression of the four lamprey *meis* genes during embryogenesis, by performing whole mount in-situ hybridization. We detected differential onset of expression of the four genes in the developing neural plate, with *meisB* preceding the other genes (Fig. 2A). *meisB* is first visible from st17 in a broad domain with an anterior limit within the presumptive hindbrain territory. *meisD* expression is detected starting at st18 in bilateral domains adjacent to the neural plate. By st19, all genes display expression in overlapping domains within the neural tube, which become more prominent at st20. At these stages, *meisA* and *meisB* neural expression is broader along the A-P axis than that of *meisC* and *meisD*, which are restricted to more anterior domains in the presumptive hindbrain. Expression adjacent to the neural plate/tube is also seen for *meisA* and *meisB*.

At later stages of lamprey development, during head extension and pharyngeogenesis, all four genes exhibit complex and dynamic expression patterns in the developing neural tube (Fig. 2B). At st22-23, during hindbrain segmentation, *meisA*, *meisB* and *meisD* each have segment-like stripes of expression in the hindbrain, consistent with rhombomere (r)-specific domains. At these stages, *meisC* expression is restricted to the midbrain and posterior axial levels of the

neural tube. At later stages, from st24-26, each gene is expressed in the hindbrain in partially overlapping domains.

To resolve the hindbrain expression domains and their relationship to rhombomeric segments more clearly by in-situ hybridization, we focused on st23.5 embryos and compared expression domains to those of previously characterized marker genes *wnt1* and *krox20* (*egr2*) (Murakami et al., 2004; Parker et al., 2019a) (Fig. 3). At this stage, the lamprey hindbrain is overtly segmented, with *hox* genes expressed in rhombomeric domains and at least six rhombomeres visible by gene expression (Parker et al., 2014a, 2019a). *wnt1* is strongly expressed in the midbrain abutting the midbrain-hindbrain boundary and at lower levels in the dorsal hindbrain (Fig. 3A), and *krox20* marks r3 and r5 (Fig. 3B). We compared expression of individual genes in dorsal views of separate embryos side-by-side, to evaluate the specific rhombomeric expression domains (Fig. 3A-F). To validate these interpretations, we also performed a series of double in-situ analyses using combinations of *meis* and marker gene expression (Fig. 3G-I, K-N). Since two color in-situ hybridizations often lead to weak and diffuse staining of the second color in our hands in lamprey, we developed both probes with the same color, selecting appropriate marker genes in each case such that expression domains do not overlap with the *meis* expression being characterized. The otic vesicle is adjacent to r4 at this stage, which provides a morphological feature as an additional avenue for inferring rhombomere identities.

Using these markers and the otic vesicle as a landmark, we determined that *meisD* is expressed in r2-r6 with varying intensity in individual segments, but is not expressed in r1, as delineated by *wnt1* in the midbrain (Fig. 3 F,I,N). *meisC* expression is located in the midbrain at this stage, based on comparison with *krox20* in r3/r5, and is detectable at low levels in the hindbrain (Fig. 3E,H,M). *meisA* expression is observed in both the midbrain and hindbrain, precluding the use of *wnt1* and *krox20* marker comparison. However, domains could be resolved by their relation to the otic vesicle and other morphological landmarks, revealing a midbrain domain that abuts the midbrain-hindbrain boundary, and segmental stripes of expression in r2, r3 and r4 (Fig. 3C,K). Similarly, *meisB* exhibits clear segmental expression in r2, r3 and r4, as shown by comparison with *wnt1* midbrain expression (Fig. 3F, I, N). Thus, *meisA* and *meisB* share expression in r2-r4 domains but only *meisA* is expressed in the midbrain. All four genes also show expression at more posterior levels of the neural tube, near the junction between the posterior-hindbrain and anterior spinal-cord. Our analyses reveal that each gene has distinct patterns of rhombomeric expression (Fig. 3J). None of them display

expression in r1, and two of them (*meisA* and *meisC*) display similar expression in the midbrain abutting the midbrain-hindbrain boundary.

Dynamic pharyngeal expression domains are also observed for each *meis* gene (Fig. 2B). From st21-26, the lamprey pharynx is progressively segmented into a series of arches and pouches, ultimately leading to 8 pharyngeal arches at st26. To resolve these pharyngeal expression domains, we generated frontal sections on st23-26 embryos after whole mount in-situ hybridization (Fig. 4). Since the signals from individual *meisA* and *meisB* probes are relatively low at st23-24, extending times for detection of the color reaction leads to diffuse signals in internal tissues. Thus, we used a *meisA/B* exonic probe to capture the expression domains of both *meisA* and *meisB* simultaneously (labelled as *meisA/B*). Frontal sections reveal *meisA/B*, *meisC* and *meisD* each to have distinct expression patterns in the pharynx, with partial overlaps in domains between genes. *meisD* expression is initially detected in the pharyngeal endoderm and mesoderm at st23, with an anterior limit at approximately the level of the PA2/PA3 border. In contrast, *meisC* expression is initially detectable in the neural crest posterior to PA1. *meisA/B* gene expression is seen in a complex pattern across multiple tissues, including ectoderm and mesenchymal tissues at this stage, with an anterior limit in PA2. These domains persist through st24 for each gene, but each also starts to be expressed in PA1 as development progresses. By st26, *meisD* is strongly expressed in mesenchymal domains of PA1 and PA2, with weaker ectodermal expression. *meisA/B* and *meisC* expression is observed in the mesenchyme of each pharyngeal arch, and *meisA/B* expression is also detectable in the ectoderm.

In summary, our in-situ analyses reveal that each of the four lamprey *meis* genes display temporally and spatially dynamic patterns of expression, some of which partially overlap. Several of these expression domains are transiently coupled to segment-specific regions in the developing hindbrain and select pharyngeal arches. The overlap and diversity in expression patterns between genes suggests considerable complexity in the regulatory inputs that govern patterns of expression of *meis* genes in head development.

3.3. Conserved Hox/TALE-bound elements in lamprey *meis* loci

Previous studies in jawed vertebrates have identified *Meis* enhancers that are bound by Hox and TALE factors, implicating these factors in the regulation of *Meis* gene expression (Amin et al., 2015; De Kumar et al., 2017a; Ladam et al., 2018; Parker et al., 2019b; Parker et al., 2011;

Parker et al., 2014b). As a first step toward addressing whether such Hox and TALE regulatory inputs into *meis* expression may also be present in lamprey, we asked whether any of these known Hox/TALE-responsive *Meis* enhancers could be identified by sequence conservation in the lamprey genome.

We previously demonstrated that a *Meis2* downstream element (which we have named *Element 1* in the present work) is bound by Hoxa1, Meis and Pbx factors in a mouse embryonic stem cell line differentiated into a neural fate (Fig. 5A) (De Kumar et al., 2017a). This element in the mouse locus is conserved downstream of a zebrafish *meis2* ortholog, and when the mouse *Meis2 Element 1* is tested for enhancer activity in a transgenic zebrafish reporter assay, it mediates GFP expression in r2-r7 and in PA2-7 (De Kumar et al., 2017a). Another highly conserved element further downstream of *Meis2* (*Element 2*) was identified in a separate study and shown to mediate reporter expression in r3/r4 and PA2 in zebrafish embryos (Parker et al., 2011; Parker et al., 2014b). This element is bound by Hoxa2, Meis and Pbx in mouse PA2 and we find that Hoxa1, Meis and Pbx also occupy this site in differentiated ES cells (Fig. 5A). Interestingly, we have found that both *Meis2 Element 1* and *Element 2* have paralogous counterparts in the mouse *Meis1* locus, although neither have paralogs in the *Meis3* locus (Fig. 5). We harnessed the previously published genome-wide binding data for Hoxa1, Meis and Pbx factors in neural cell culture (De Kumar et al., 2017a), and for Hoxa2, Meis and Pbx in mouse PA2 (Amin et al., 2015; Donaldson et al., 2012), to ascertain whether these transcription factors also bind to the paralogous *Meis1* elements (Fig. 5A). This analysis revealed an enrichment for occupancy of these factors over *Element1* and *Element2* from both *Meis1* and *Meis2* loci, suggesting a shared role for Hox and TALE factors in regulating both *Meis1* and *Meis2* in mouse.

The *Meis1* and *Meis2* genes in mouse are paralogs that resulted from an ancient duplication event that is thought to have occurred in early vertebrates (Simakov et al., 2020). Hence, the similarly positioned conserved sequences across each locus, which correspond to putative paralogous regulatory elements, implies that they may be derived from ancient elements that predated this duplication event. Since one or more whole genome duplication events are thought to have occurred prior to the divergence of jawed and jawless vertebrate lineages (Simakov et al., 2020; Smith and Keinath, 2015), this suggests that such elements may have been present in ancestral vertebrates and may be conserved in lamprey. Indeed, the zebrafish *meis2 Element 2* was originally identified and characterized based on its conservation in lamprey (McEwen et al., 2009; Parker et al., 2011), although it could not be linked definitively to

a lamprey *meis* gene at the time due to the fragmentary nature of the early lamprey genome assembly. Thus, we sought to identify the lamprey *meis* gene to which this homolog of *Element 2* is associated and to examine if *Element 1* and additional paralogs of *Element 2* are also conserved in other lamprey *meis* genes.

We searched for these elements in the lamprey genome using BLAST and by a more sensitive multiple alignment approach (MLAGAN) focusing on *meis* genomic loci. The homology to *Element 2* in mouse *Meis2* had previously been identified in an assembly of the lamprey somatic genome (Smith et al., 2013), situated between the exons of a neighboring gene – *c15orf41*. Searching for *Element 2* in the most recent lamprey germline genome (Smith et al., 2018), which is more complete and contains longer contigs, we were able to identify the homologous element within the *c15orf41* locus, adjacent to *meisB*, confirming its association with a lamprey *meis* gene (Fig. 5B). Furthermore, we also identified homologous sequences related to this same distal element downstream of *meisC* and *meisD*, suggesting they are paralogous regulatory elements present in all of the lamprey and mouse *meis* loci, apart from *Meis3* (depicted in Fig. 5B). In searching for the *Meis2 Element 1* in lamprey, we identified a homologous sequence downstream of *meisD*, but did not find homologs associated with any other lamprey *meis* genes (Fig. 5B). In the hagfish genome, we identified homologs of *Element 2* downstream of both *meis* gene loci (Fig. 5B). A homolog of *Element 1* was found downstream of *meis 6940*, in keeping with its inferred homology to *meisD*. It is notable that the positions of these elements relative to each other and to their associated *Meis* genes are conserved across species, while the distances between them vary. This may reflect a high level of selection to retain the relative position and order of these elements within the various *Meis* loci.

The *Meis2 Element1* and *Element2* have previously been shown to contain consensus Pbx-Hox and *Meis* transcription factor binding sites required for their enhancer activities (De Kumar et al., 2017a; Parker et al., 2011). Pbx-Hox dimers have been shown to bind octameric motifs with the consensus TGATNNAT, where the variable core 'NN' influences which specific Hox paralogs can bind to the sequence (Mann et al., 2009), while *MEIS* and *PREP* factors preferentially bind a CTGTCA hexameric sequence (Chang et al., 1997; Penkov et al., 2013). Genome-wide binding analyses in mouse embryos have found the octomeric motif to be a common sequence feature within Pbx-*Meis*-*Prep* co-bound regions, with these factors presumably forming complexes with Hox factors at these sites. While the majority of vertebrate Hox factors have not

had their genome-wide binding properties characterized *in vivo*, recent studies in mouse have confirmed that Hoxa2 and Hoxa3 bind genomic regulatory sites that contain octomeric motifs (Bridoux et al., 2020; Donaldson et al., 2012). These factors display flexible sequence preferences within the core region and a slight preference for GAC adjacent to the 3' end of the TGATNNAT motif (Fig. 6B, Fig. 7). To search for conservation of these regulatory motifs, we aligned the lamprey homologs of these elements with their mouse counterparts, identifying a high degree of conservation of these sites between all sequences in the alignments (Fig. 6B, Fig. 7). Thus, the Hoxa2, Pbx and Meis binding observed for these elements in mouse (Figure 6A) is likely to be mediated through these Pbx-Hox and Meis sites, and the conserved sites in the lamprey elements may also receive equivalent/similar regulatory inputs. The identified Pbx-Hox sites exhibit diversity in the variable core sequence between sites and between species, with dinucleotide combinations of AA, GA and TA present, which may influence their responsiveness to specific Hox and TALE factors.

The alignments revealed that *Element 2* is differentially conserved across lamprey paralogs. All paralogs show conservation of a 5' fragment (*Element 2A*), but only the *meisB* and *meisD* paralogs display conservation in a 3' region (*Element 2B*). Additionally, the distances between *Elements 2A* and *2B* vary among paralogs, as measured from the conserved Pbx-Hox motif in *Element 2A* to the 5' conserved Meis motif of *Element 2B* (Fig. 6B). This distance is very similar between the *Meis1*, *Meis2*, and *meisA/B* paralogous elements, at approximately 250-280bp, which implies it may be under a selective constraint to retain the proximity of these sites for functional activity. The distance is much larger for the *meisD* elements, 1572bp, suggesting a relaxation of this constraint in this instance. Thus, the Meis loci differ in their retention of certain fragments of *Element2* and in the relative spacing between the fragments, with the intervening sequences diverging between loci, which may influence the functional output of these elements. In summary, the conservation of Pbx-Hox and Meis binding site motifs and the evidence for binding of Hox, Pbx and Meis factors to these elements in mouse suggests that regulatory inputs from Hox and TALE factors through these sites may also be important in contributing to regulation of the lamprey *meis* genes.

3.4. Enhancer activity of *meis* conserved non-coding elements in lamprey

The gnathostome *Meis* elements have previously been tested for enhancer activity by reporter assay in zebrafish embryos. The mouse homolog of *Meis2 Element 1* was shown to activate GFP expression in r2-r7 and in PA2-7 (Fig7) (De Kumar et al., 2017a). A zebrafish homolog of *Meis2 Element 2a* drives reporter expression in r3-4 and PA2 neural crest, while *Element 2b* is also active in the hindbrain (Parker et al., 2011). We tested the mouse *Element 2* from *Meis1* and *Meis2* for enhancer activity in zebrafish, confirming that they generate similar domains of GFP expression in the anterior hindbrain and PA2 (Fig. 7). To address whether the 4 conserved lamprey elements identified in the alignments can function as tissue-specific enhancers, we cloned them into the *HLC GFP* reporter vector (Parker et al., 2014b) and tested them in a transgenic assay in lamprey embryos. We found that each element drives expression in distinct domains of the neural tube, with striped, segment-like patterns visible at st23-24 (Fig. 7). *meisD Element 1* activates GFP expression in the posterior hindbrain, at approximately the r4-r6 level. *meisA/B Element 2* up-regulates GFP in the anterior hindbrain, at the level of r2-r4, while *meisD Element 2* mediates expression across domains that encompass r2-r6. In contrast, the *meisC* homolog, which only has sequence conservation with the *Element 2a* sub-fragment, does not mediate GFP expression in the hindbrain, but generates a domain of expression in the midbrain. While the gnathostome enhancers direct expression in neural crest domains, no such activity was observed for any of the lamprey enhancers, suggesting that other elements are required for this activity.

The domains of enhancer activity of each element in the hindbrain are in good agreement with the endogenous expression domains of their associated *meis* genes. This is illustrated by the schematic dorsal views comparing the endogenous in-situ and transgenic reporter patterns in Fig. 7. It is interesting to note that the paralogous *Element 2* enhancers in lamprey each direct reporter activity in distinct domains of the neural tube despite containing homologous Hox and TALE binding site motifs. This raises the possibility that sequence differences within these sites, or differences in neighboring sequences, may contribute to the diverse activities of these elements. Such sequence variations could potentially mediate altered interactions with Hox, TALE and other transcription factors to modify regulatory activity. Our analyses suggest that the presence of conserved *Meis*, *Pbx* and *Hox* binding sites in these lamprey enhancers links *Hox* and *TALE* factors to regulation of lamprey *meis* genes in the developing hindbrain, indicating a

deep ancestry for these regulatory interactions prior to the divergence of jawed and jawless vertebrates.

4. Discussion

We have identified four *meis* genes in the developing sea lamprey and characterized their expression in the head, with a focus on the hindbrain and pharynx. This represents an initial step toward investigating regulatory interactions between *meis* and *hox* genes in lamprey. We have found that *meis* expression patterns are coupled to rhombomeric domains in the developing hindbrain and characterized dynamic expression in various tissues during pharyngeogenesis. Direct upstream regulatory inputs from Hox and TALE (Meis and PBX) proteins have been identified in control of several mouse *Meis* genes. We searched the lamprey genome and identified and characterized homologous *cis*-elements associated with each lamprey *meis* gene. Sequence comparisons indicated a series of conserved binding motifs for Hox- and TALE proteins in the mouse and lamprey genes. Regulatory assays demonstrated that these elements act as neural enhancers in lamprey embryos, directing reporter expression in appropriate domains when compared to expression of their associated endogenous *meis* gene. The regulatory activity and conservation of Hox and TALE binding sites in these elements, reveals that ancient Hox and TALE-responsive enhancers regulated expression of ancestral vertebrate *meis* gene/s in segmental domains in the hindbrain, and have been retained in *meis* loci during vertebrate evolution. These findings raise a number of interesting points related to the function and regulation of *meis* genes and the evolution of gene regulatory networks that control hindbrain segmentation and axial patterning in vertebrates.

4.1. Lamprey *meis* repertoire and relationship to gnathostome *Meis* genes

We identified four *meis* genes in lamprey, two of which are tandem duplicates. Interestingly, a pair of tandem duplicates are also present in hagfish, but not in jawed vertebrate genomes, which suggests the duplication event giving rise to this tandem pair occurred in a jawless vertebrate ancestor, prior to the lamprey-hagfish divergence. Given that jawed vertebrates such as mammals and sarcopterygian fish have three *meis* genes (Amemiya et al., 2013; Nakamura et al., 1996), this suggests that there may have been three *meis* genes in an ancestral

vertebrate genome prior to the divergence of jawed and jawless vertebrate lineages. However, it is known that additional genome/chromosomal duplication events have shaped the lamprey genome (for example, it has 6 *Hox* clusters) (Mehta et al., 2013; Smith et al., 2013; Smith et al., 2018). Furthermore, recent chromosome-scale gene synteny analyses support a model in which all extant vertebrates share a common whole genome duplication event (1R), with subsequent independent whole genome or chromosomal duplications then occurring in the early jawed vertebrate and lamprey lineages (Simakov et al., 2020; Smith and Keinath, 2015). Under this scenario, the ancestor of lamprey and gnathostomes is likely to have had two *meis* genes. Since our phylogenetic analyses group *meisC* with *Meis3*, and *meisA,B,D* with *Meis1,2*, this could imply that an ancestral 1R vertebrate genome contained a *Meis* gene ancestral to *meisC/Meis3*, and another ancestral to *meisA,B,D/Meis1,2*. However, the relatively low bootstrap support for the *meisC-Meis3* grouping makes this a tentative interpretation, and there were no obvious patterns of conserved synteny between the *Meis* loci of lamprey and mouse that could provide further support. Thus, we cannot confidently infer the precise phylogenetic relationships between *meis* homologs in lamprey and jawed vertebrates from the present analysis.

4.2. *Meis* gene expression in relation to *Hox* genes

Given that regulatory links between *meis* and *hox* genes play important roles during hindbrain and pharyngeal patterning in gnathostomes (Amin et al., 2015; De Kumar et al., 2017a; Parker et al., 2019b), we sought to investigate whether such interactions may also shape development of the lamprey head. Through characterization of *meis* gene expression in the developing hindbrain and pharynx of lamprey we found that each gene is expressed in complex and temporally-dynamic patterns in these tissues. These genes exhibit discreet expression patterns, but also share common domains, especially at later stages. The first expression we detected is in the neural plate at st17 (*meisB*), which is very similar to the earliest *hox* expression displayed by the *hox PG1* genes (Parker et al., 2019a). At later stages, during hindbrain segmentation (st21-24), *meisA*, *meisB* and *meisD* transiently show prominent stripes of rhombomeric expression, which then become less defined due to expansion of expression domains after st24 into broader territories. This dynamic period of segmental *meis* expression corresponds to the developmental stages during which *hox* genes are most clearly expressed in rhombomeric domains in lamprey (Parker et al., 2014a, 2019a). Indeed, it is noteworthy that the *hox*-negative

rhombomere 1 is also the only rhombomere with no *meis* expression at this stage. In gnathostomes, *meis* genes are also expressed posterior to r1 during early hindbrain development (Sanchez-Guardado et al., 2011; Waskiewicz et al., 2001), and are required for correct Hox-dependent hindbrain patterning (Choe et al., 2002; Waskiewicz et al., 2001) through numerous regulatory inputs into Hox-responsive hindbrain segmental enhancer elements (De Kumar et al., 2017a; Parker et al., 2019b; Parker and Krumlauf, 2017, 2020). Thus, the observed temporal overlap between segmental *hox* and *meis* gene expression in lamprey is in keeping with the idea that Hox-Meis interactions may also be employed during lamprey hindbrain segmental patterning. This could occur through regulation of *hox* genes by Meis factors, regulation of *meis* genes by Hox factors, or a combination of both, establishing regulatory feedback circuits that reinforce robust and stable domains of segmental expression.

In addition to expression domains in the embryonic nervous system, we also identified expression of each gene in the developing pharynx in distinct tissue-specific domains. For instance, *meisC* is initially restricted to the NC-derived pharyngeal mesenchyme posterior to PA1. It is noteworthy that expression of the other *meis* paralogs, such as *meisA/B* in the pharyngeal mesenchyme and *meisD* in the endoderm and mesoderm, is also initially restricted to PA2 and posterior. Early *hox* expression in the developing pharynx is similarly restricted to PA2 and posterior. For example, *hoxβ1* is detected in endoderm and ectoderm, and *hoxa2* in NC is posterior to PA1 (Parker et al., 2019a). We have previously shown that *meisC* expression is tightly correlated with the onset of *hoxa2* in NC at st22-23. This led us to speculate that, as shown for *Meis2* in mouse, *meisC* may contribute to regulation of *hoxa2* in the neural crest through binding to a conserved upstream hindbrain/NC enhancer which contains Hox, Pbx and Meis binding sites. Hence, the general overlap of *meis* and *hox* expression patterns in these PA domains may reflect a series of regulatory interactions between the *hox* and *meis* genes. It is also important to note that many domains of *meis* expression do not overlap with *hox* expression, as illustrated by expression in anterior regions, such as the midbrain and PA1. Furthermore, in gnathostomes *meis* genes have also been shown to display hox-independent functions (Lopez-Delgado et al., 2021; Moens and Selleri, 2006), suggesting that the expression domains in lamprey which do not overlap with *hox* expression are associated with other roles in embryonic and tissue development. Future functional perturbation studies will be required to address the nature of *meis* gene functions in the pharynx and potential regulatory interactions with *Hox* genes and other factors.

In non-vertebrate deuterostomes, the coincident expression of Hox and Meis genes in *Saccoglossus* (Aronowicz and Lowe, 2006; Lemons et al., 2010) and amphioxus neuroectoderm (Albuixech-Crespo et al., 2017; Schubert et al., 2006) previously led us to speculate that these factors may have comprised an ancestral deuterostome regulatory circuit involved in neuroectodermal patterning (Parker et al., 2019b). Thus, auto- and cross-regulatory interactions within this network may be conserved between vertebrates and deuterostomes. Future comparative genomic and *cis*-regulatory analyses with invertebrate deuterostomes may enable the pan-vertebrate *Meis* hindbrain enhancers to be traced deeper in the deuterostome lineage.

4.3. Lamprey *meis* enhancers

Comparisons between lamprey and jawed vertebrate genomes have identified hundreds of conserved non-coding elements that represent putative enhancers inherited from a vertebrate common ancestor (McEwen et al., 2009; Parker et al., 2011). Previous analyses identified a cluster of such enhancers downstream of *meis2* in gnathostome genomes, within the exons of *c15orf41*, revealing that they have enhancer activity within the hindbrain in zebrafish and lamprey. These elements have conserved motifs for Pbx-Hox and Meis binding sites, suggesting deep conservation of these auto and cross-regulatory inputs into *Meis* genes across vertebrates. However, the fragmentary nature of the lamprey genome assembly at the time left it unclear whether these elements were associated with a *meis* gene in lamprey. Subsequent studies have surveyed genome-wide transcription factor binding of Hox, Meis and Pbx factors in mouse, identifying key inputs of these factors into *Meis* enhancers (Amin et al., 2015; De Kumar et al., 2017a; Donaldson et al., 2012; Ladam et al., 2018). Here, we have integrated these findings to build upon our knowledge of the lamprey *meis* enhancers. We find that the *Meis2 Element 2* from jawed vertebrates is conserved in lamprey and associated with the *meisA/B* locus. This region has regulatory activity and possesses a cluster of Hox, Pbx and Meis binding motifs. Furthermore, we have discovered additional paralogs of this enhancer associated with *meisC* and *meisD*. We extended these comparisons and detected a homolog of another known Hox- and TALE-responsive enhancer (*meis2 Element 1*) downstream of lamprey *meisD*. These elements all show high conservation of Pbx-Hox and Meis binding site motifs. This suggests that Hox and TALE factors have a common role in providing regulatory inputs on the activity of these *meis* elements in lamprey, by analogy to their roles for the homologous mouse enhancers.

We have verified the enhancer activity of each of these conserved elements in lamprey and find their domains of reporter activity to be in good agreement with key aspects of the expression domains in the hindbrain of their associated *meis* genes. Together with the conserved Pbx-Hox and Meis binding sites and the known regulatory inputs of those factors into the homologous enhancers in mouse, this strongly implicates Hox and TALE factors in regulation of the rhombomeric expression observed for lamprey *meis* genes.

The conserved Meis enhancers that we have identified comprise an interesting example of a set of orthologous, co-orthologous and paralogous *cis*-regulatory elements at various evolutionary distances from each other, including the huge distance between jawed and jawless vertebrates. Thousands of conserved non-coding elements have been identified among jawed vertebrate genomes and hundreds of these are conserved in lamprey (Hara et al., 2018; McEwen et al., 2009; Smith et al., 2013; Woolfe et al., 2005). However, only few of these CNEs have been characterized in detail with respect to their gene regulatory activities during vertebrate embryogenesis (Papdogiannis et al., 2020; Parker et al., 2011; Parker et al., 2014b). Thus, our analysis of this set of *cis*-elements in enhancers of Meis genes presents an interesting opportunity to glean insights into the functional evolution of *cis*-regulatory modules across large evolutionary timescales, enabling comparison of elements between species and within species (i.e. between paralogs) in the vertebrate phylum. These findings may also apply more broadly to other regulatory elements in vertebrate genomes. Here, we focus on two striking features of these *Meis* elements – their high sequence conservation despite diverging functional outputs, and their conserved positions within the *Meis* gene loci.

Despite sharing conserved Pbx-Hox and Meis sites in equivalent locations and orientations within each element, the paralogous lamprey *Element 2* enhancers each drive discreet patterns of reporter expression. These differences may be due to small sequence differences within the conserved motifs, which could influence binding of specific combinations of Hox and TALE factors, or to differences in adjacent sequences that bind factors that impact or modulate their activity. We previously found that highly conserved elements in the mouse and lamprey *Hox2* genes mediate distinct regulatory outputs despite having virtually identical Hox, Pbx and Meis binding sequences (Parker et al., 2019b). Understanding the mechanistic basis of these contextual differences in regulatory activity of paralogous elements is an important area for future investigation. This diversity of activity from paralogous elements has been observed for other highly conserved enhancers and may reflect sub-functional partitioning or evolution of novel regulatory activities after enhancer duplication (Goode et al., 2011). It has been

suggested that the flanking sequences around core CNE domains could be recruited to influence regulatory function over time in lineage- or paralog-specific manners (Polychronopoulos et al., 2017). Such exploitation and elaboration of existing regulatory potential in regions flanking ancient, conserved enhancer ‘cores’ has been characterized in a fish *Sox21* lens enhancer (Pauls et al., 2015). Two recent studies have subjected specific enhancers to systematic mutations and analyzed how these small sequence variations influence their spatial pattern and activity levels in reporter assays in flies (Fuqua et al., 2020; Le Poul et al., 2020). These studies revealed widespread pleiotropic effects for the majority of mutations, showing that these developmental enhancers contain densely packed regulatory information along their sequence, beyond that appreciated from simple conservation or limited functional analyses. This could constrain their evolvability between species in instances where their functional output is under selective constraint, while also providing evolutionary potential in situations when enhancers are duplicated, such as the whole genome duplication/s in early vertebrates. Our reporter analyses on the lamprey and jawed vertebrate *Meis* CNEs appear to support this model, since the elements have conserved cores, containing Pbx-Hox and *Meis* motifs, divergent flanking sequences, and drive diverse patterns of reporter activity.

In addition to their high sequence conservation, the *Meis* elements also exhibit a striking positional conservation relative to *Meis* genes. CNEs have been shown to reside in clusters that often span genomic regions containing developmental genes and are maintained in syntenic blocks, which have been named genomic regulatory blocks (Kikuta et al., 2007). Genome-wide studies have shown that genomic regulatory blocks often fall within TADs – genomic regions within which frequent chromosomal interactions occur (Harmston et al., 2017). This has been demonstrated for the *Meis2* locus, with boundaries of CNE density being concordant with the TAD boundaries (Polychronopoulos et al., 2017). Mounting evidence implicates TADs with roles in genome organization and function, while disruptions within TADs have been associated with changes in gene expression (Beagan and Phillips-Cremins, 2020; Lupianez et al., 2015). TADs associated with genomic regulatory blocks are often conserved across species, which may be important for maintaining appropriate regulation of gene expression (Harmston et al., 2017). Given their positional overlap, it is tempting to speculate that CNEs may play a role in the formation or maintenance of TADs, though there is no clear evidence for or against this idea. If so, the positional conservation of the *Meis* CNEs in this study may reflect selective pressure for maintenance of appropriate genomic architecture and gene regulation. Thus, these elements may have further regulatory contributions beyond those visible by the reporter assay approach

that we have used to characterize their functions. These could include roles in determining chromatin structure and influencing the activity of neighboring regulatory elements within the genomic regulatory blocks that encompass the *Meis* loci. In this regard, it would be interesting to knock out or transpose these elements within and between their genomic regulatory blocks, in order to investigate how this influences the chromosomal interactions within *Meis* loci and the regulation of *Meis* gene expression.

It is noteworthy that despite the mouse *Meis* enhancers displaying activity in neural crest and being bound by *Hoxa2* and TALE factors in mouse PA2, their lamprey homologs do not mediate reporter expression in lamprey neural crest cells. This may reflect lineage-specific functional diversification of these enhancers between lamprey and jawed vertebrates and that other elements are required for mediating expression in neural crest cells. Thus, these *meis* elements provide evidence for conservation of ancestral activity in mediating segmental *meis* expression in the hindbrain, but it is not clear whether their activity in neural crest cells was ancestral or emerged in the jawed vertebrate lineage. Further studies are required to provide more mechanistic details linking Hox and TALE factors to regulation of *meis* in the lamprey neural crest. Towards this end it will be interesting to perturb Hox/TALE functions in lamprey and characterize additional lamprey *meis* enhancers. Given the complexity of *meis* gene expression during development, it appears likely that there are more *meis* enhancers underlying their regulation in lamprey that remain to be identified.

4.4. Conclusion

In conclusion, our analysis suggests that ancient Hox and TALE-responsive enhancers regulated expression of ancestral vertebrate *Meis* genes in segmental domains in the hindbrain and have been retained in *Meis* loci during vertebrate evolution. The presence of conserved *Meis*, *Pbx* and *Hox* binding sites in these lamprey enhancers links Hox and TALE factors to regulation of lamprey *meis* genes in the developing hindbrain, indicating a deep ancestry for these regulatory interactions prior to the divergence of jawed and jawless vertebrates. Looking deeper in chordate evolution, this study provides a platform for regulatory comparisons with non-vertebrate deuterostomes (Minor et al., 2019), to investigate how *Hox* regulatory networks evolved to pattern the vertebrate head.

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Figures:**Figure 1: *meis* genes in the sea lamprey genome.**

(A) A schematic depicting the three *meis* gene-containing genomic loci from the sea lamprey germline genome assembly (kPetmar1). Predicted protein-coding genes are shown as black arrows, non-coding genes (lncRNA) as grey arrows, with the arrowhead indicating the direction of transcription. Positions of predicted exons are shown as vertical lines. Chromosomal positions of the genomic loci are given. (B) A schematic diagram showing *Meis* genomic loci and neighboring genes from lamprey, hagfish and mouse. Genes are denoted by arrowheads, with direction indicating their direction of transcription. *Meis* gene colors indicate inferred homology between lamprey and hagfish. (C-D) Phylogenetic analysis of the sea lamprey *Meis* proteins. Four predicted full length sea lamprey *Meis* protein sequences were aligned with selected vertebrate *Meis* homologues, including (C) or excluding Hagfish (D), with *ciona* *Meis* as an outgroup. The trees generated by Maximum Likelihood are shown, based on 642 positions (C) and 617 positions (D) in the alignment. Support for each node is shown as a percentage from 500 bootstrap replicates. The trees are drawn to scale, with the unit for evolutionary distance being the number of amino acid substitutions per site. Lamprey genes are highlighted (blue shading). Abbreviations for species names are given on the right-hand side of the tree.

Figure 2: Expression of lamprey *meis* genes in the developing head.

(A) Dorsal views of st17-st20 embryos with expression of *meis* genes revealed by *in situ* hybridisation. The anterior (a), posterior (p), left (l) and right (r) sides are annotated in the top-left image. (B) Expression of *meis* genes in st21-26 embryos, shown in lateral view with anterior to the left. The locations of the midbrain (mb) and hindbrain (hb) are indicated on the *meisA* embryos for reference.

Figure 3: Rhombomeric expression of lamprey *meis* genes.

Dorsal (A-I) and lateral (K-N) views of st23.5 lamprey embryos are shown. (A-F) In-situ hybridisation against *wnt1*, *krox20*, and *meisA-D* genes reveal midbrain and hindbrain segments in the neural tube. (G,L) A double in-situ hybridization against *wnt1* and *meisB*, showing segmental *meisB* expression in the hindbrain posterior to the r1/r2 boundary, in r2-r4. *wnt1*

marks the caudal limit of the midbrain (mb), revealing the midbrain-hindbrain boundary (mhb). (H,M) *meisC* is expressed in the midbrain and posterior hindbrain/spinal cord, while *krox20* marks r3 and r5. (I-N), *meisD* shows stripes of expression in r2-r6, with lower expression levels in the neural tube posterior to r6, as compared to *wnt1* in the midbrain. (J) A depiction of a dorsal view of a st23.5 lamprey embryo, summarizing the segmental gene expression domains in the neural tube shown in (A-F).

Figure 4: Pharyngeal expression of lamprey *meis* genes.

Frontal sections of st23, st24 and st26 embryos reveal pharyngeal expression domains of *meis* genes. Sections are oriented with anterior to the top. For *meisA* and *meisB*, an exonic in situ probe hybridizing to both genes was used. Schematic frontal sections shown on the right, indicating different tissue layers. Pharyngeal arches are numbered (1-8). ec, ectoderm; en, endoderm; m, mouth; me, mesenchyme; n, notochord; nt, neural tube.

Figure 5: Hox/TALE-bound *Meis* enhancers are conserved between mouse and lamprey.

(A) DNA-binding profiles for Hox and TALE factors in neural cell culture and pharyngeal arch 2 tissues at the mouse *Meis2* and *Meis1* genomic loci. Open chromatin regions (ATAC) are also shown for mouse neural cell culture. Genes are annotated (top), with directions of transcription indicated (arrows). Previously characterized Hox/TALE-dependent hindbrain/NC enhancers from the *Meis2* locus (*Element1* and *Element2*), and their paralogs in the *Meis1* locus, are highlighted in purple. (B) Summary of sequence conservation of *Element1* and *Element2* across *Meis* gene loci in mouse, lamprey and hagfish. Positions of genes are indicated (arrows) and locations of homologs of *Element1* (white rectangle) and *Element2* (blue rectangle) are shown. Lamprey and hagfish *meis* genes are colored as in Fig.1B to indicate inferred homology. The distance of the conserved element from the end of the associated *meis* gene are given in kilobases (kb) below each element. The *C15orf41* gene downstream of *Meis2* in mouse has a homolog downstream of *meisB* in lamprey and hagfish but is not conserved in the other *Meis* gene loci in mouse, lamprey or hagfish.

Figure 6: Hox/TALE binding sites are conserved between mouse and lamprey.

(A) DNA-binding profiles for Hox and TALE factors at the mouse *Element 1* and *Element 2* in *Meis* loci. Sequence conservation with homologous loci from mouse and lamprey are shown in Vista plots below the DNA-binding profiles, with conserved regions represented as pink peaks. These conserved sequences align to the regions of Hox/TALE binding, as shown (highlighted in purple). Based on sequence conservation, *Element 2* is sub-divided into two adjacent sub-sequences: *Elements 2A* and *2B*. *Element 2A* abuts an exon of *c15orf41* downstream of mouse *Meis2* and lamprey *meisB*. *Element 2B* was not identifiable in the lamprey *meisC* locus. (B) Sections of sequence alignments for *Element 1* and *Element 2* from mouse and lamprey *Meis* gene loci, revealing short conserved sequence blocks (asterisks below alignments). Consensus binding motifs for Meis and Pbx-Hox factors are shown as sequence logos below the alignments, and conserved sequences that correspond to these motifs are indicated in the alignments, with arrows denoting the orientation of motifs. The distances between *Element 2A* and *Element 2B* are given for each gene locus.

Figure 7: Conserved Hox/TALE binding site sequences match known binding preferences.

(A) Short conserved sequence blocks from *Elements 1* and *2* are aligned above motifs characterised for HOXA2 and HOXA3 bound sites (Bridoux et al., 2020), and Meis-Prep-Pbx bound sites (Penkov et al., 2013). (B) Conserved instances of Meis motifs from *Elements 1* and *2* are shown above a characterised Meis1 binding motif (Chang et al., 1997).

Figure 8: Activity of homologous Meis enhancers in zebrafish and lamprey.

(A) Lateral view of a 30hpf embryo from a transgenic zebrafish reporter line showing GFP expression driven by *Meis2 Element 1* in the hindbrain and neural crest. (B,C) Lateral views of 54hpf transient transgenic zebrafish embryos expressing GFP in the hindbrain and neural crest, driven by *Meis1 Element 2* (B) and *Meis2 Element 2* (C). For *Meis1 Element 2*, of 72 embryos screened, 30 displayed hindbrain GFP expression and 10 had NC expression. For *Meis2 Element2*, of 30/64 embryos had hindbrain expression and 7/64 showed NC expression. For the lamprey enhancers, lateral (D,G,J,M) and dorsal (E,H,K,N) views of st23-24 transient transgenic lamprey embryos are shown. Endogenous expression of *meis* genes is shown in dorsal views of whole-mount embryos for comparison with reporter expression domains (F,I,L,O). (D,E) *meisD*

Element 1 drives GFP expression in the hindbrain (n = 44/81), at approximately the r4-r6 level. (G,H) *meisA/B Element 2* up-regulates GFP in the anterior hindbrain (n = 82/104) at the level of r2-r4. (J,K) *meisC Element 2* is active in the midbrain (n = 39/105). (M,N) *meisD Element 2* drives GFP broadly in the hindbrain (n = 84/103) at the levels of r2-r6. (P) A depiction summarizing the segmental domains of enhancer activity of the lamprey *meis* enhancers shown in (D-N) in relation to the expression domains of *meis* genes. hb, hindbrain; mb, midbrain, nc, neural crest.

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A **B** **C** **D**

A **B**

I Summary of hindbrain segmental gene expression

A **B**

A

Gene	Element/Site	Sequence
mouse Meis1	1	GTGATAAATGAC
mouse Meis2	1	TTGATAAATGAC
lamprey meisD	1	GTGATAAATGAG
mouse Meis1	2a	ATGATGAATGTC
mouse Meis2	2a	ATGATGAATGTC
lamprey meisA/B	2a	ATGATGAATGTC
lamprey meisC	2a	ATGACGAATGTC
lamprey meisD	2a	ATGATGAATGTC
mouse Meis1	2b site1	AAGATTAATGGT
mouse Meis2	2b site1	ATGATAAATGAC
lamprey meisA/B	2b site1	ATGATGAATGGC
lamprey meisD	2b site1	ATGATGAATGGG
mouse Meis1	2b site2	ATGATGAATGAC
mouse Meis2	2b site2	ATGATAAATGAC
lamprey meisA/B	2b site2	ATGATAAATGAC
lamprey meisD	2b site2	ATGATAAATGAC

HOXA3, HOXA2 motifs:
Bridoux *et al* (2020)

HOXA3
HOXA2

Meis-Prep-Pbx 'OCTA' motif:
Penkov *et al* (2013)

B

Gene	Element/Site	Sequence
mouse Meis1	1	CACTGTCACAG
mouse Meis2	1	CAATGTCACCC
lamprey meisD	1	CACTGTCACGC
mouse Meis1	2a	ACCTGTCATGG
mouse Meis2	2a	ACCTGTCACCC
lamprey meisA/B	2a	AACTGTCAGGA
lamprey meisC	2a	ACCTGTCAGT
lamprey meisD	2a	GGCTGTCACCA
mouse Meis1	2b site1	TGCTGTCATGG
mouse Meis2	2b site1	CACTGTCAGTG
lamprey meisA/B	2b site1	CCCTGTCAGGG
lamprey meisD	2b site1	CACTGTCACCC
mouse Meis1	2b site2	GCCTGTCAGAT
mouse Meis2	2b site2	CTCTGTCAGCA
lamprey meisA/B	2b site2	CCCTGTCAGTG
lamprey meisD	2b site2	CCCTGTCAGCG
mouse Meis1	2b site3	ACCTGTCAGCG
mouse Meis2	2b site3	ACCTGTCATCT
lamprey meisA/B	2b site3	ACCTGTCACCG
lamprey meisD	2b site3	ACCTGTCCGCG

Meis1 motif:
Chang *et al* (1997)

P

Summary of hindbrain segmental enhancer activity and *meis* gene expression

Highlights

- The Sea Lamprey genome has four *meis* genes
- Expression of the *meis* genes is coupled to hindbrain segments and pharyngeal arches
- Lamprey and jawed vertebrate *meis* loci contain conserved enhancers
- Vertebrate *meis/Meis* enhancers contain conserved Hox, Pbx and Meis binding sites
- Ancient Hox and TALE-responsive enhancers regulate segmental *meis* expression