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# Evidence from cyclostomes for complex regionalization of the ancestral vertebrate brain

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**The vertebrate brain is highly complex, but its evolutionary origin remains elusive. Because of the absence of certain developmental domains generally marked by the expression of regulatory genes, the embryonic brain of the lamprey, a jawless vertebrate, had been regarded as representing a less complex, ancestral state of the vertebrate brain. Specifically, absence of a *Hedgehog*- and *Nkx2.1*-positive domain in the lamprey subpallium was thought to be similar to mouse mutants where suppression of *Nkx2.1* leads to a loss of the medial ganglionic eminence (MGE)[1,2]. Here we show that the brain of the hagfish, another cyclostome group, develops domains equivalent to the MGE and rhombic lip, resembling the gnathostome brain. Moreover, further investigation of lamprey larvae revealed that these domains are also present, ruling out the possibility of convergent evolution between hagfish and gnathostomes. Thus, brain regionalization as seen in crown gnathostomes is not an evolutionary innovation of this group, but dates back to the latest vertebrate ancestor prior to the divergence of cyclostomes and gnathostomes more than 500 million years ago (mya).**

During development, the vertebrate brain exhibits highly organized regionalization along the dorsoventral and anteroposterior axes[3,4]; the entire neural tube is divided dorsoventrally into basal and alar plates, medially united by the roof plate dorsally, and by the floor plate ventrally. Antero-posteriorly, the brain primordium is divided into a series of 'neuromeres', the boundaries of which serve as scaffolds for axonal growth, leading to conserved patterns of nerve tracts (**Fig. 1a,b** and **Extended Data Fig. 1**). Each domain is determined through specific and regionalized gene expression to differentiate into a functionally specialized region. This brain regionalization, with distinct domains marked by differential, specific gene expression

patterns (or genoarchitecture[4]), is surprisingly conserved in the gnathostome embryos examined so far (**Fig. 1a,b**), showing that the basic developmental plan of this organ has a deep evolutionary origin and continues to serve as a blueprint for brain development across the gnathostomes. However, it remains unclear when this developmental programme emerged in evolution.

Previous studies on the brain of the lamprey, one of the two groups of cyclostomes together with the hagfish[5], have suggested that this might represent a pre-gnathostome state of brain evolution, mainly because of the absence of two key domains: (i) the pallidum, which is involved in motor control and develops from a *Nkx2.1*-positive region within the subpallium in gnathostomes, the MGE[4] (**Fig. 1a,c**); and (ii) a morphologically distinct cerebellum, which plays crucial roles in sensory integration and motor planning, as well as complex cognitive processing in gnathostomes[6] (**Fig. 1a,c**). The absence of such structures in the lamprey has placed the origin of the complete vertebrate brain architecture, with an MGE and cerebellum, at the base of the gnathostome lineage[2,7]. However, GABAergic interneurons, which in gnathostomes migrate from the embryonic MGE and pallidum-like domains, have been observed in juvenile and adult lampreys[8–10] (**Fig. 1d**), raising the possibility that the lamprey's embryonic brain represents a secondary reduction from the ancestral one[7].

To address this question, we studied a member of a different group of cyclostomes, the inshore hagfish (*Eptatretus burgeri*). The adult brain of the hagfish has a greatly reduced brain ventricle and exhibits highly differentiated organization of the pallium, which makes it difficult to compare with that of other vertebrates[11] (**Extended Data Fig. 2a,b**). Moreover, as is the case for the lamprey, neither a pallidum nor a cerebellum has been identified in the hagfish[12,13]. Identification of embryonic domains

in the hagfish brain has also been difficult, mainly because of the distortion apparent in fixed specimens[14–16]. To overcome these problems, we obtained embryos of *Eptatretus burgeri* at Bashford Dean stages 45 and 53[17,18] (**Extended Data Fig. 2c–h**), and compared brain development with lampreys, as well as with the cloudy catshark, *Scyliorhinus torazame*, as a gnathostome outgroup to cyclostomes.

Observations of the developing hagfish brain (**Fig. 2**) prompted us to consider putative homologies of hagfish brain regionalization with those of other vertebrates. At stage 53, the hagfish brain clearly shows well-differentiated domains with conspicuous ventricles similar to those in gnathostomes (**Fig. 2h**), which we describe here according to the prosomeric model[3]. In gnathostomes and the lamprey, transverse commissures serve as landmarks for the identification of neuromeres[11,19,20] (**Fig. 1a–c**). Longitudinal nerve tracts, such as the medial longitudinal fasciculus and some commissures—including the optic chiasm and the habenular and posterior commissures—allowed us to mark the regionalization of the hagfish embryonic brain. In this, the habenular commissure defines the roof of the thalamus, or prosomere 2 (p2)[3], and the posterior commissure marks the posterior boundary of the pretectum (p1)[4,11] (**Figs 1** and **3a,h**). The ventral limit of the telencephalon is defined as dorsal to the optic chiasm (**Fig. 3a**). The interbulbar commissure, already described in the lamprey[11], is also present in the hagfish (**Fig. 3a,h**). The latter commissure is equivalent to the gnathostome pallial or hippocampal commissures in the telencephalon, and crosses secondary olfactory fibres as in the lamprey[11]. Finally, the commissure lying posterior to the midbrain–hindbrain boundary of the hagfish brain corresponds to the *commissura vestibulo-lateralis*, which consists of the eighth nerve and lateral line nerve fibres as in the lamprey, although in the hagfish the cerebellar commissure has not been identified[6] (**Fig. 3a**). Throughout the

103 developmental stages examined, we could identify no overt epiphysis in the hagfish,
104 although this has been described in the lamprey and jawed vertebrates (**Extended Data**
105 **Fig. 5**).

106 To test for the presence or absence of neuromeres, we investigated the brain
107 genoarchitecture of the hagfish embryo to evaluate its regionalization. The
108 telencephalon of the hagfish embryo could be identified as a domain expressing *FoxG1*,
109 *EmxB* and *Pax6* orthologs[21,22] (**Fig. 3c–e**,**j–l**, **Extended Data Figs 3c–e,j,k** and **11**). The
110 hypothalamus was located rostral to the diencephalon, identified by the co-expression of
111 one hagfish *hedgehog* gene, *Hh2* (one of the three hagfish *Hh* genes newly identified in
112 this study, named *Hh2–4*, and distinct from our previously reported hagfish *Hh1*[18]; see
113 **Supplementary Methods** and **Extended Data Figs 7** and **10**), and *Nkx2.1/2.4,*
114 orthologous to gnathostome *Nkx2.1* genes (**Fig. 3f,g**, **Extended Data Figs 3f,g** and **10**).
115 However, we could not determine whether the anterior end of the floor plate is
116 hypothalamic or not[23]. As in the lamprey[2], hagfish *Hh2* expression also marked rather
117 clearly the position of the *zona limitans intrathalamica* (*zli*) between the prethalamus
118 (p3) and thalamus (p2). This position had not been identified previously (**Extended**
119 **Data Fig. 6**). Given the *Shh*-dependent inductive activity of *zli*[24], its presence is
120 consistent with the p2/p3 boundary in the hagfish diencephalon as noted above. More
121 caudally, the pretectum (p1), which is identified by the posterior commissure, expressed
122 *Pax6*, as in gnathostomes[4] (**Fig. 3a,k** and **Extended Data Fig. 3a,l**). Thus, in the
123 hagfish, we clearly identified the hypothalamus and diencephalic prosomeres (p1;
124 pretectum, p2; thalamus and p3; prethalamus) rostral to the midbrain, with the *zli*
125 between p2 and p3. Finally, the mesencephalon was defined as a *Pax6*-negative region
126 caudal to the posterior commissure (**Fig. 3a,h,k** and **Extended Data Fig. 3a,l**).

Strikingly, and unlike in lampreys[25], we detected in the stage 53 *E. burgeri* embryo an independent and conspicuous expression domain of *Nkx2.1/2.4* dorsal (rostral according to conventional columnar model[4]) to the optic chiasm, corresponding to the region where the gnathostome MGE would form[22] (**Fig. 3g**). Moreover, *Hh2* was also expressed in a slightly restricted area of this *Nkx2.1/2.4*-positive domain, possibly corresponding to a part of the MGE and preoptic area[4] (**Fig. 3f**). Therefore, the putative MGE in the hagfish arises as a conspicuous region within the brain, located in the rostral part of the telencephalon (ventral according to the conventional columnar model[4]), and possibly associated with the lateral ganglionic eminence (LGE) posterior to it (**Fig. 3m**).

In modern gnathostomes, the cerebellum differentiates from the dorsal part of rhombomere l, whereby granular cells are supplied from the rhombic lip to constitute a layer of the cerebellar cortex[4]. The rhombic lip in gnathostomes expresses *Pax6,* which enables granular cells to migrate[26]. Therefore, regional specification of the rhombic lip is a prerequisite to acquisitioning of the cerebellum. However, an overt cerebellum is missing in cyclostomes, although there are fibres possibly equivalent to commissures posterior to the midbrain–hindbrain boundary in gnathostomes[6] (**Fig. 1**). In the lamprey, previous studies have deduced the absence of a rhombic lip-derived cerebellum from the lack of *Pax6* expression in the corresponding area[2]. Unlike in the lamprey, the hagfish rhombencephalon clearly develops a *Pax6*-positive rhombic lip, as in gnathostomes (**Fig. 4a–d**). This was confirmed by the expression of a different rhombic lip marker, *Atoh1*, which is involved in the development of excitatory cells in the gnathostome cerebellum in the hagfish dorsal hindbrain[27] (**Extended Data Fig. 9**).

Our results show that the hagfish and gnathostome embryonic brains share a

common regional patterning, leading us to two scenarios: (i) the hagfish/gnathostome genoarchitecture represents an ancestral programme for all vertebrates, and lampreys have lost the MGE and rhombic lip secondarily; alternatively, (ii) the ancestral brain developmental pattern did not have an MGE or rhombic lip, which were acquired independently in the lineages of hagfish and gnathostomes by convergent evolution. To clarify this, we have reinvestigated brain development of the Japanese lamprey, *Lethenteron japonicum,* with a recently reported draft genome sequence[28]. We found two extra lamprey *Nkx2.1* orthologous genes (*Nkx2.1/2.4B* and *Nkx2.1/2.4C*, see **Online Methods,** and **Extended Data Figs 10** and **13** and **Extended Data Table 1**), as well as one extra *Hedgehog* paralog, *HhD*, in addition to the previously reported *Nkx2.1* (renamed as *Nkx2.1/2.4A*) and *HhA-C* genes[7] (**Extended Data Figs 8** and **10**). Surprisingly, lamprey *Nkx2.1/2.4B* and *C* were expressed in a rostral domain within the subpallium, suggesting the presence of an MGE in the lamprey (**Fig. 4g,h** and **Extended Data Fig. 8**). On the other hand, no *HhD* expression was detected (**Extended Data Fig. 8**), suggesting that—although present—specification of the MGE in the lamprey is still somehow divergent compared with other vertebrates.

Next, we looked for the presence of a rhombic lip in *L. japonicum* larvae. Lamprey *Atoh1*, *Wnt1*, *Ptf1a* genes, as well as the recently identified lamprey *Pax6B*[29] were expressed in a dorsoventrally organized pattern in the hindbrain (**Fig. 4**), implying specification of a rhombic lip-like property as in gnathostomes, although *Pax6B* expression was found more ventrally than in gnathostomes (**Extended Data Fig. 9s**). Therefore, as in the case of the MGE, lamprey rhombic lip specification is somehow divergent from the gnathostome condition. Interestingly, because gnathostome *Atoh1* and *Ptf1a* are involved in the development of the cerebellum granule and Purkinje cells,

175 respectively[27], certain genetic backgrounds underlying the acquisition of the cerebellum
176 proper in gnathostomes, yet absent in cyclostomes, might have been established before
177 the splitting of the two groups (**Fig. 4**). Altogether, the lamprey developmental pattern
178 suggests the presence of a rhombic lip domain (**Fig. 4** and **Extended Data Fig. 9**).
179 The absence of certain domains in the lamprey brain once led researchers to
180 examine a scenario in which the brain evolution followed a stepwise elaboration toward
181 crown gnathostomes. However, we show here that two major domains of the vertebrate
182 brain, previously regarded as gnathostome novelties, are indeed present in cyclostomes
183 as distinct gene expression domains. These domains would have followed further
184 subsequent elaboration in each lineage. For instance, only the gnathostome rhombic lip
185 led to the cerebellum proper as a gnathostome novelty (**Fig. 4j**). That part would
186 probably have preceded acquisition of a jaw, because jawless stem gnathostomes, such
187 as ostracoderms, likely had a morphologically distinct cerebellum[30]. Thus, our findings
188 dramatically alter the accepted evolutionary scenario (**Fig. 4**) and imply that the
189 vertebrate brain genoarchitecture, with regional gene expression patterns as seen in
190 crown gnathostomes, is much older than previously thought, dating back to more than
191 500 mya when the latest common ancestor of crown vertebrates arose (**Fig. 4**).

192 **METHODS SUMMARY**

193 Embryos of *Eptatretus burgeri* were collected and staged as described[16–18] and fixed
194 with 4% paraformaldehyde. Three-dimensional images of the ventricular system of
195 hagfish embryos were reconstructed with Avizo (Visualization Sciences Group,
196 Bordeaux, France) by using the dataset in ref. 18. Axons were stained with an anti-
197 acetylated tubulin antibody (Sigma-Aldrich). Images were recorded with a DP70 digital
198 camera (Olympus Corp., Tokyo, Japan) attached to a light microscope (Olympus

BX60).

**Online content**

Any additional **Methods**, **Extended Data** display items and **Source Data** are available in the online version of the paper; references unique to these sections appear only in the online paper.


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**AUTHOR CONTRIBUTIONS**

F.S., J.P.-A., Y.M. and S. Kuratani designed the experiments and wrote the paper. F.S., J.P.-A., Y.O., S.A., N.A. and T.H. performed molecular works. N.S. discussed and

interpreted the data. S. Kuraku performed the molecular phylogenetic analysis. W.T. performed the *Nkx2.1/2.4* locus synteny conservation analysis.

**AUTHOR INFORMATION**

The sequences of the genes described in this study have been deposited in the NCBI (National Center for Biotechnology Information) GenBank under accession nos. LC028239-43, LC028245 and KT897926-27 and KT897930-38. Reprints and permissions are available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed S. Kuratani (saizo@cdb.riken.jp).

## METHODS

**Sample collection and aquarium maintenance of hagfish, lamprey and shark.** Mature males and females of the Japanese inshore hagfish, *Eptatretus burgeri*, were obtained in the Japan Sea off Shimane prefecture as described[31]. Embryos were deposited in a cage set on the sea bottom in October 2011. Deposited eggs were then collected and incubated in an aquarium until embryos reached the required stages. Embryos were fixed with 4% paraformaldehyde in late March 2012. We mainly used two embryos (at stages 45 and 53) in this study. Embryos used in **Fig. 4d** and **Extended Data Figs 9m** and **9n** were obtained previously[16,18]. Sources of the Japanese lamprey, *L. japonicum* (recently included as a synonym for *L. camtschaticum*), were as described[32]. Lamprey embryos were staged as described[33] and fixed in 4% paraformaldehyde. Adult catsharks, *S. torazame*, were obtained in Ibaraki prefecture, Japan, and kept in aquarium tanks. Deposited eggs were incubated in the same tanks at 16 °C until embryos reached the required stages. Catshark embryos were staged and fixed with 4% paraformaldehyde as described[34]. The sampling and experiment were conducted according to the institutional and national guidelines for animal ethics.

**Gene identification, cDNA cloning and sequencing.** For the identification of hagfish *Hedgehog*, *Atoh1* and catshark *Atoh1*, *Ptf1a* and *Wnt1* genes, the nucleotide sequences were obtained from embryonic transcriptomes of *E. burgeri* and *S. torazame*, respectively, and assembled from an in-house RNA-seq dataset (to be published elsewhere). Hagfish *FoxG* sequence was retrieved from the Vertebrate Time Capsule database[35]. In all cases, we performed TBLASTN using as queries the amino acid sequences of corresponding orthologs from human, mouse, chicken and zebrafish (plus

329 *L. japonicum HhA*, *-B, -C* and the amphioxus *Branchiostoma floridae Hh* gene in the
330 case of hagfish *Hedgehog*). We found four putative hagfish *Hh* genes, including the
331 previously reported *Hh1*[18], and designated the three new genes as *Hh2–4* arbitrarily. The
332 Lamprey *Nkx2.1/2.4, Atoh1, Ptf1a* and *Wnt1* genes were identified by means of
333 TBLASTN using human, mouse, chicken and zebrafish counterparts as queries against
334 *L. japonicum* low-coverage draft genome sequence version LetJap1.0[28] (accession no.
335 APJL00000000, http://jlampreygenome.imcb.a-star.edu.sg/). We found three *Nkx2.1* or
336 *Nkx2.4* gene-containing scaffolds: 242 (GenBank accession no. KE993913, containing
337 the previously reported TTF-1[36]), 17 (KE993688) and 73 (KE993744). We found
338 lamprey *Wnt1* in scaffold 103 (KE993774), two *Ptf1a* paralogs in scaffolds 196 (*Ptf1a-*
339 *A*; KE993867) and 473 (*Ptf1a-B*; KE994144), and *Atoh1* in scaffold 280 (KE993951).
340 The corresponding gene sequences were predicted by means of GeneWise[37]
341 (http://www.ebi.ac.uk/Tools/psa/genewise/) or GENSCAN[38]
342 (http://genes.mit.edu/GENSCAN.html) and curated manually. Phylogenetic and synteny
343 conservation analyses (see below) could not resolve the orthology relationships between
344 *Nkx2.1* and *Nkx2.4*, so we designated these genes as *Nkx2.1/2.4A* (TTF-1, scaffold 242),
345 *Nkx2.1/2.4B* (scaffold 17) and *Nkx2.1/2.4C* (scaffold 73). As in gnathostomes[39],
346 *Nkx2.1/Nkx2.4A* and *C* were linked to the *Nkx2.2* and *Nkx2.8* genes, which were
347 designated as *Nkx2.2/2.8A* and *C*, respectively. A partial sequence of a third putative
348 *Nkx2.2/2.8B* gene was found in contig 79345 (accession no. APJL01110193). The shark
349 *FoxG1* homologous gene was cloned using degenerate primers designed based on the
350 consensus amino acid sequences of vertebrate *FoxG1* counterparts (forward primer, 5′–
351 ATGGGNGANMGNAARGA–3′; reverse, 5′–NCCYTGRTTYTGRTGNGT–3′). An *E.*
352 *burgeri OtxC* gene fragment was obtained using specific primers based on the

nucleotide sequence of the Atlantic hagfish, *Myxine glutinosa, MgOtxC*[40].

Total RNAs of *E. burgeri* and *S. torazame* were extracted from whole embryos using TRIZOL reagent (Invitrogen Life Sciences). Reverse transcription polymerase chain reaction (RT–PCR) was performed to amplify fragments of each gene with specific primers. Lamprey gene fragments were obtained from a stage-24 cDNA library (ZAP-cDNA Synthesis Kit, Cat. #200400, Stratagene). PCR products were cloned into the pCRII-TOPO vector (Invitrogen) and subsequently sequenced with a 3130 Sequence Analyzer (Applied Biosystems). The 5′ and 3′ ends were amplified with GeneRacer kits (Invitrogen Life Sciences), or from the lamprey cDNA library with customized primers. The cDNA sequences identified here have been deposited in GenBank under accession numbers LC028239-43, LC028245 and KT897926-27 and KT897930-38. Shark *Pax6* (AB773851), *Dlx2* (AB293582), *Shh* (AB247650), *Nkx2.1* (AB773852), hagfish *Pax6* (AB270704), *EmxA* (AB935431), *EmxB* (AB935432), *Nkx2.1* (AB747372), *Hh1* (AB703680) and *Fgf8/17* (AB703681) and lamprey *Pax6* (AB061220), *Pax6B* (Feiner et al., 2014, Supplementary Table S3[29]), *DlxA* (AB292628), *HhA* (AB124584)*, B* (AB583549) and *C* (AB583548) were obtained previously[7,18,25,41–43].

***In situ* hybridization.** *In situ* hybridization on paraffin wax-embedded sections was performed as described[32]. Briefly, deparaffinized sections (8–10 μm) were treated with Protease K (2–10 μg/ml) at room temperature for 5–15 min. Slides were incubated in hybridization buffer (50% formamide, 5 × SSC (pH 7.0), 1% SDS, 50 μg/ml total yeast RNA, 50 μg/ml heparin sulfate, 5 mM EDTA (pH 8.0), 0.1% CHAPS and 1% blocking reagent (Roche)) with 0.2–1.0 μg/ml DIG-labelled RNA probe overnight at 65 °C. For

immunohistochemical detection, sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG Fab fragments (diluted 1:4000) for 2 h after blocking. Colour development was performed using the BM purple AP substrate (Roche). Whole-mount *in situ* hybridization of shark and lamprey embryos was performed according to refs 41 and 32, respectively.

**Immunohistochemical staining.** Deparaffinized sections of hagfish embryos were blocked with 5% skim milk in TBST (TSTM). They were then reacted overnight at room temperature with an anti-acetylated tubulin antibody (Sigma-Aldrich, T6793) diluted in TSTM (1:1000). After the samples had been washed with TBST, they were reacted for 2 h with Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen Life Sciences, A11001) diluted in TSTM (1:400). After the final wash in TBST, embryos were observed using an Axio Zoom V16 fluorescence microscope (Carl Zeiss).

**Three-dimensional (3D) reconstruction of the hagfish brain.** The 3D images of the ventricular system of hagfish embryos were reconstructed based on the dataset used in ref. 18. Reconstructed images were acquired using Avizo software (Visualization Sciences Group, Bordeaux, France).

**Molecular phylogenetic analysis.** The molecular phylogenetic trees of *Hh*, *Nkx2.1/2.4*, *Fgf8/17*, *FoxG*, *Wnt1*, *Atoh1* and *Ptf1a* genes *of E. burgeri*, *L. japonicum* and *S. torazame* were inferred with the maximum-likelihood method using PhyML3.0 (http://www.atgc-montpellier.fr/phyml/download.php) with the JTT+G4 model.

**Synteny conservation analysis.** According to ref. 44, comparisons of genomic regions including vertebrate *Nkx2.1/2.4* were performed among the following species: human (*Homo sapiens*), chicken (*Gallus gallus*), coelacanth (*Latimeria chalumnae*), western clawed frog (*Xenopus tropicalis*), elephant shark (*Callorhinchus milii*) and Japanese lamprey (*L. japonicum*). In brief, gnathostome *Nkx2.1/2.4* orthologs and the adjacent protein-coding genes were arranged using the UCSC (http://genome.ucsc.edu) and Ensembl (http://www.ensembl.org/) genome browsers. For lamprey, genome scaffolds 17 (GenBank accession no. KE993688), 73 (KE993744) and 242 (KE993913) containing the *Nkx2.1/2.4* homologs (see above) were analysed using the gene prediction tool, GENESCAN[38]. Subsequently, deduced amino acid sequences of the predicted genes were used as queries for NCBI TBLASTN searches and annotated with an E-value cut-off of $10^{-20}$.


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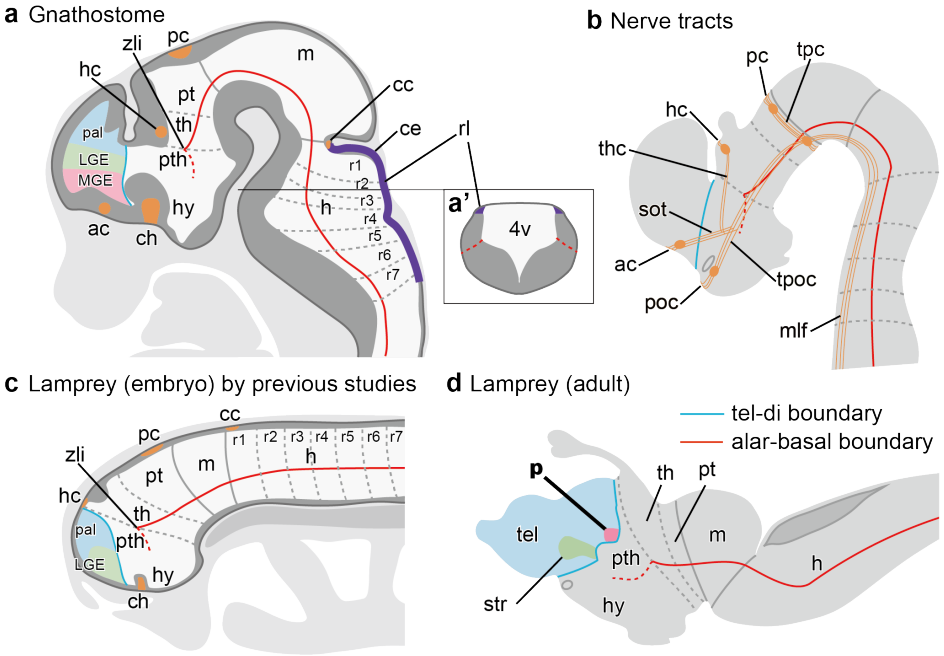


461

462 **Figure 1 | Regionalization of the vertebrate brains.** (**a**), Schematic drawing of the
463 embryonic gnathostome brain based on a mouse embryonic day (E)12.5 embryo. (**a'**), A
464 transverse section at the level of the hindbrain showing the position of the rhombic lip.
465 (**b**), Nerve tracts and commissures in the gnathostome embryonic brain based on the
466 shark embryo[19] (**Extended Data Fig. 1**). (**c**), Embryonic lamprey brain as revealed by
467 previous studies[2]. (**d**), Adult brain of the lamprey based on refs 10 and 20. Red dotted
468 lines indicate the alar plate–basal plate boundary (*sulcus limitans*). Blue lines delimit
469 the telencephalon–diencephalon boundary. Orange circles in (**c**) indicate the position of
470 cell bodies of the early nerve tracts[19]. Coloured regions in (**c**) correspond to the same
471 colours in (**d**). Note that although the pallidum equivalent region (bold "**p**" in panel (**d**))
472 was suggested to be present in the adult lamprey brain, its embryonic primordium
473 (MGE: *Nkx2.1*-positive region) has not been reported so far. Key: ac, anterior
474 commissure; cc, cerebellar commissure; ce, cerebellum; ch, optic chiasm; h, hindbrain;
475 hc, habenular commissure; hy, hypothalamus; m, midbrain; LGE, lateral ganglionic

eminence; MGE, medial ganglionic eminence; mlf, medial longitudinal fascicle; p, prospective pallidum; pal, pallium; pc, posterior commissure; poc, postoptic commissure; pt, pretectum; pth, prethalamus; r1–7, rhombomeres 1–7; rl, rhombic lip; sot, supraoptic tract; str, striatum; tel, telencephalon; th, thalamus; thc, tract of the habenular commissure; tpc, tract of the posterior commissure; tpoc, tract of the postoptic commissure; zli, *zona limitans intrathamamica*; 4v, fourth ventricle.

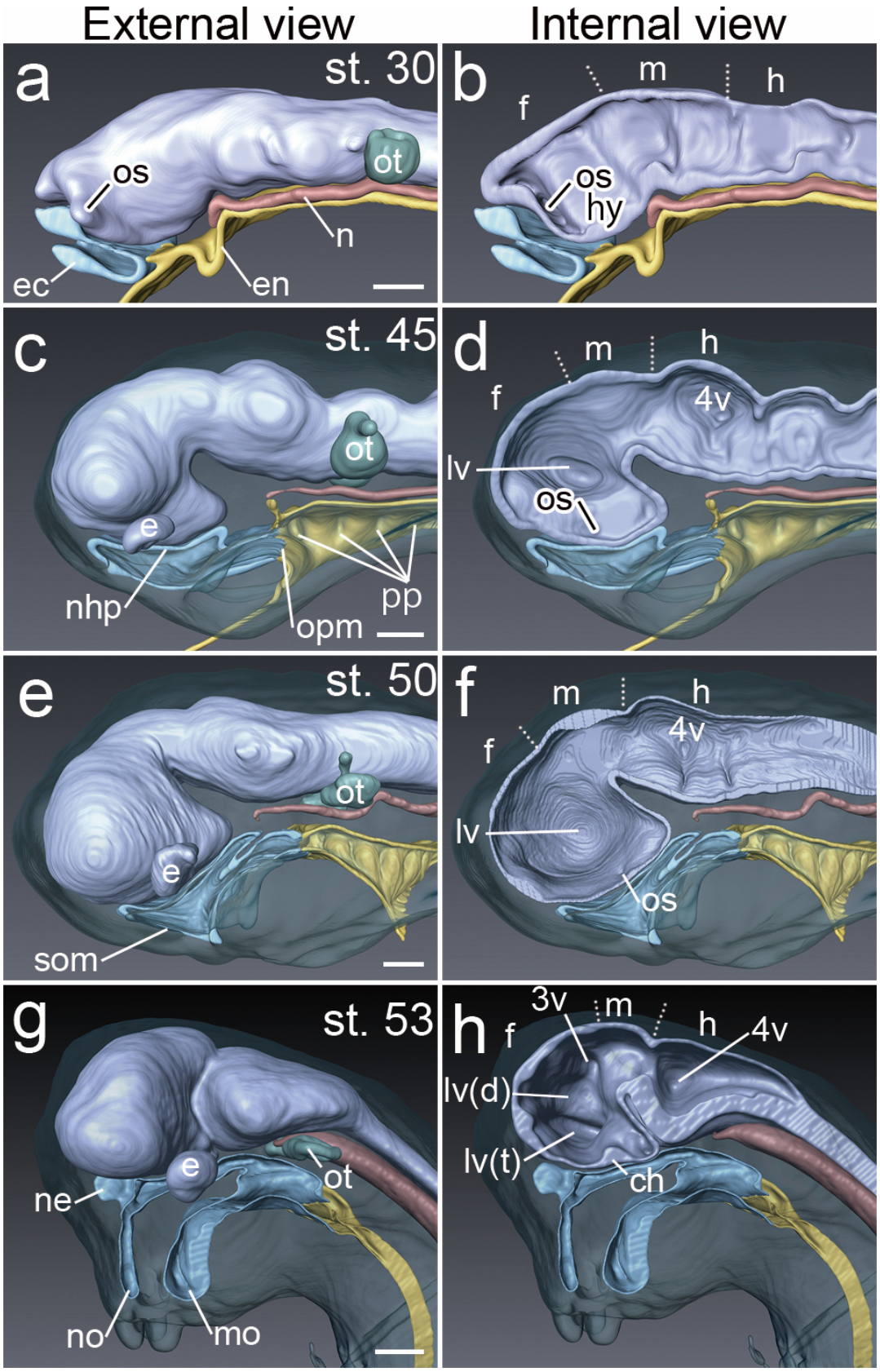
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**Figure 2 | Development of the ventricular system in the hagfish brain.** Three-dimensional images reconstructed from serial sections of *E. burgeri* embryos with Avizo software. External views (**a**, **c**, **e**, **g**) and internal views (**b**, **d**, **f**, **h**) are shown. The central nervous systems are coloured purple, the ectoderm is coloured in light blue and green, endoderm in yellow and notochord in orange. The developmental stages are based on ref. 18. Identification of brain regions is based on brain morphology, nerve staining and comparative gene expression patterns (**Fig. 3**; **Extended Data Figs 3** and **4**). At stage 53, lateral ventricles can be divided into a telencephalic part (lv(t)) and a diencephalic part (lv(d))[6]. Key: e, eye; ec, ectoderm; en, endoderm; f, forebrain; lv, lateral ventricle; lv(d), diencephalic division of lateral ventricle; lv(t), telencephalic division of lateral ventricle; mo, mouth opening; n, notochord; ne, nasal epithelium; nhp, naso-hypophyseal plate; no, nasal opening; os, optic stalk; ot, otic vesicle; opm, oropharyngeal membrane; pp, pharyngeal pouches; som, secondary oral membrane; 3v, third ventricle. See **Fig. 1** for other abbreviations. Scale bars, 200 μm.

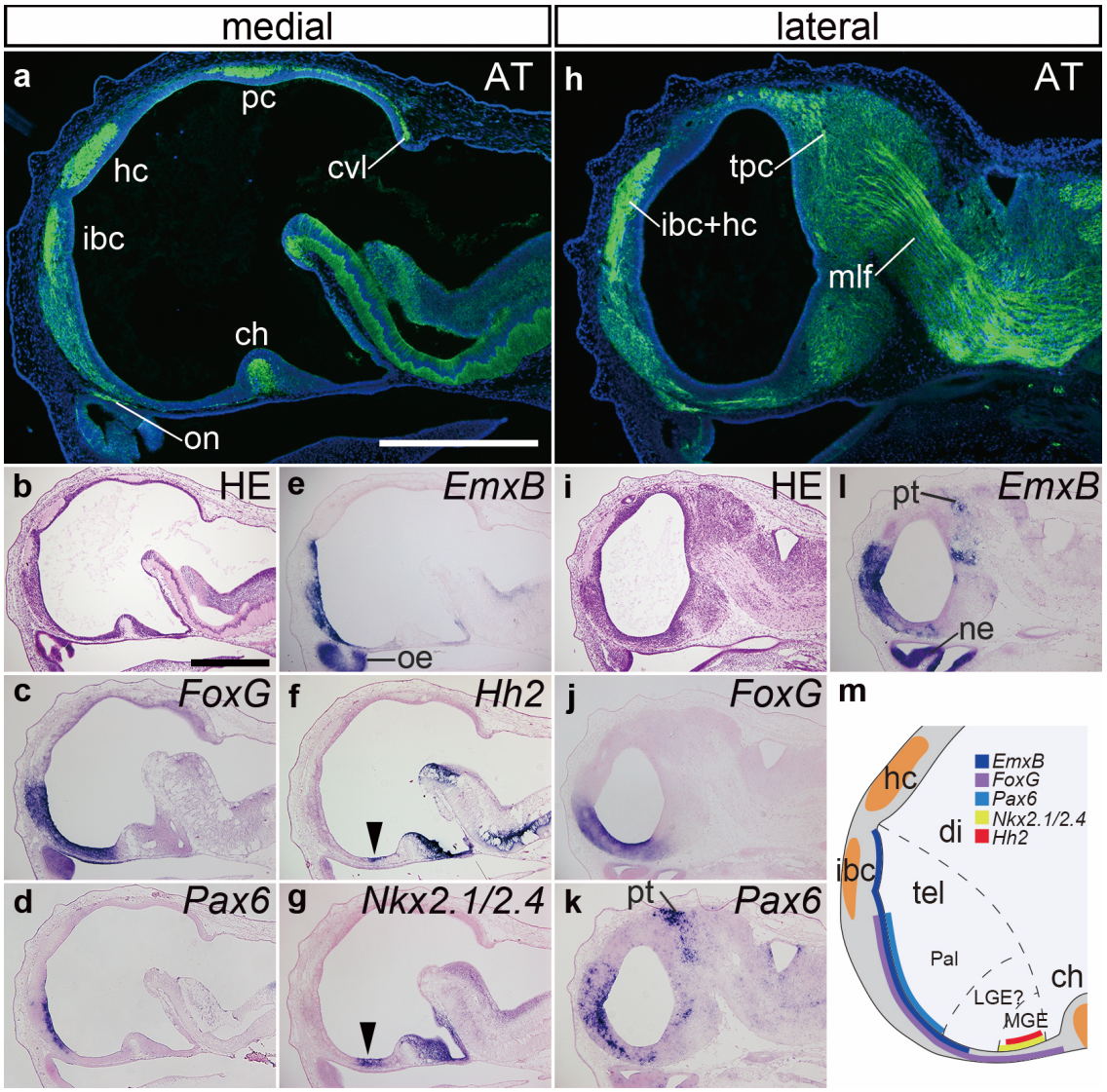
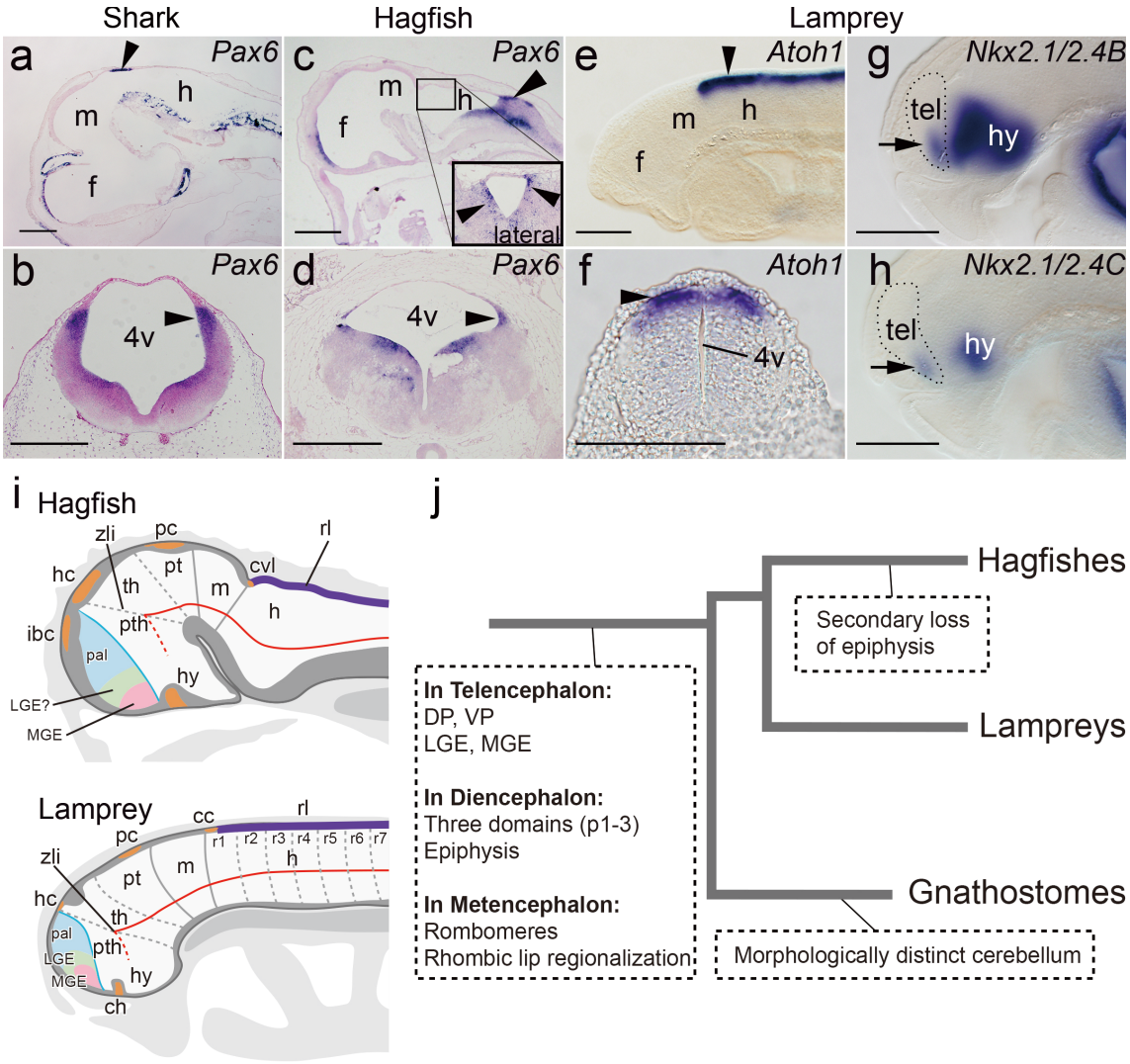



**Figure 3 | Gene expression and nervous staining in *Eptatretus burgeri* at stage 53. a**, **h**, Immunohistochemical staining of the axon bundles by anti-acetylated tubulin antibody (green) and DAPI (blue). **b**, **i**, Hematoxylin–eosin staining. **c–g, j–l**, Gene expression patterns involved in the regionalization of the forebrain. **m**, Schematic drawing of gene expressions in the telencephalon. The telencephalic territory can be identified from the expressions of *FoxG*, *Pax6* and *EmxB*[21,22]. Arrowheads in **f** and **g** indicate expressions in the MGE region of the telencephalon. Key: cvl, *commissura vestibulo-lateralis*; di, diencephalon; ibc, interbulbar commissure; on, olfactory nerve.

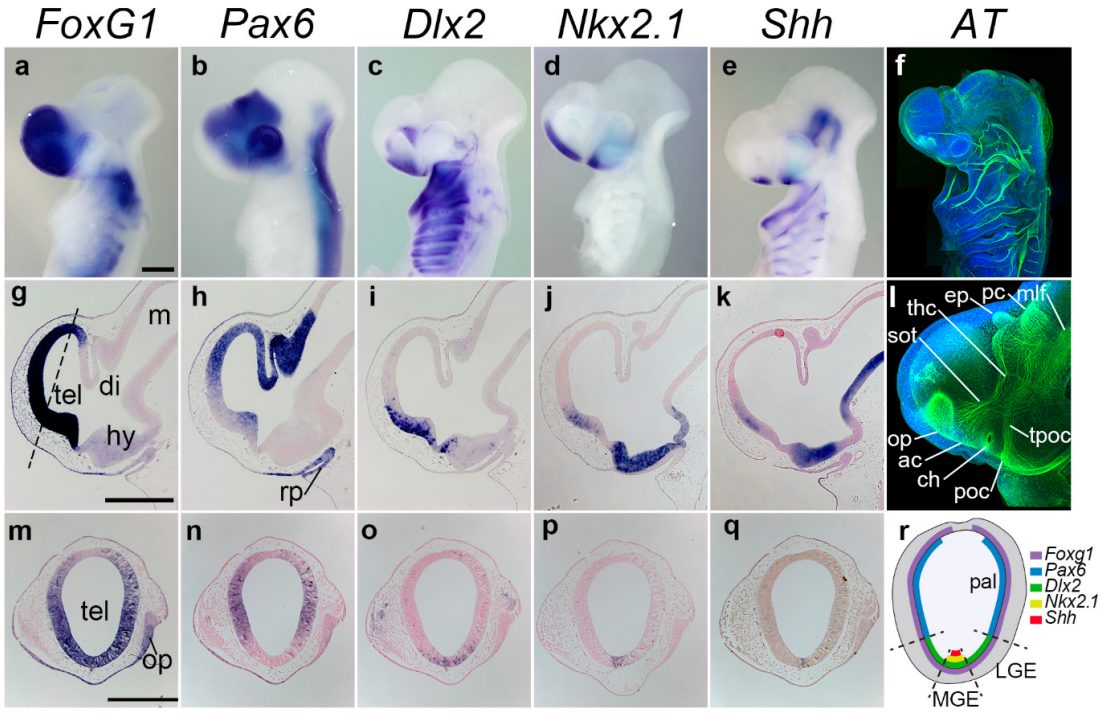
See **Fig. 1** for other abbreviations. Scale bars, 500 μm.




511

512 **Figure 4 | Presence of the rhombic lip and MGE in the hagfish and lamprey brains**
513 **and an evolutionary scenario of the vertebrate brain**. **a–f**, *Pax6* expression in the
514 hindbrain of a stage 31 catshark embryo (**a**, **b**) and in a hagfish stage 53 embryo (**c**, **d**).
515 *Atoh1* expression in a lamprey stage 26 embryo (**e**, **f**). Sagittal views (**a**, **c**, **e**) and
516 transverse sections (**b**, **d**, **f**) are shown. Arrowheads indicate expressions in the rhombic
517 lip. The inset in **c** shows rhombic lip expression on a lateral section. (**g–h**), *Nkx2.1/2.4*
518 homolog gene expression patterns in a lamprey stage 27 embryo. Arrows indicate
519 telencephalic expression of *Nkx2.1/2.4B* and *C*. (**i**), Schematic drawing of embryonic
520 hagfish and lamprey brains (stages 53 and 26, respectively) as revealed by the present

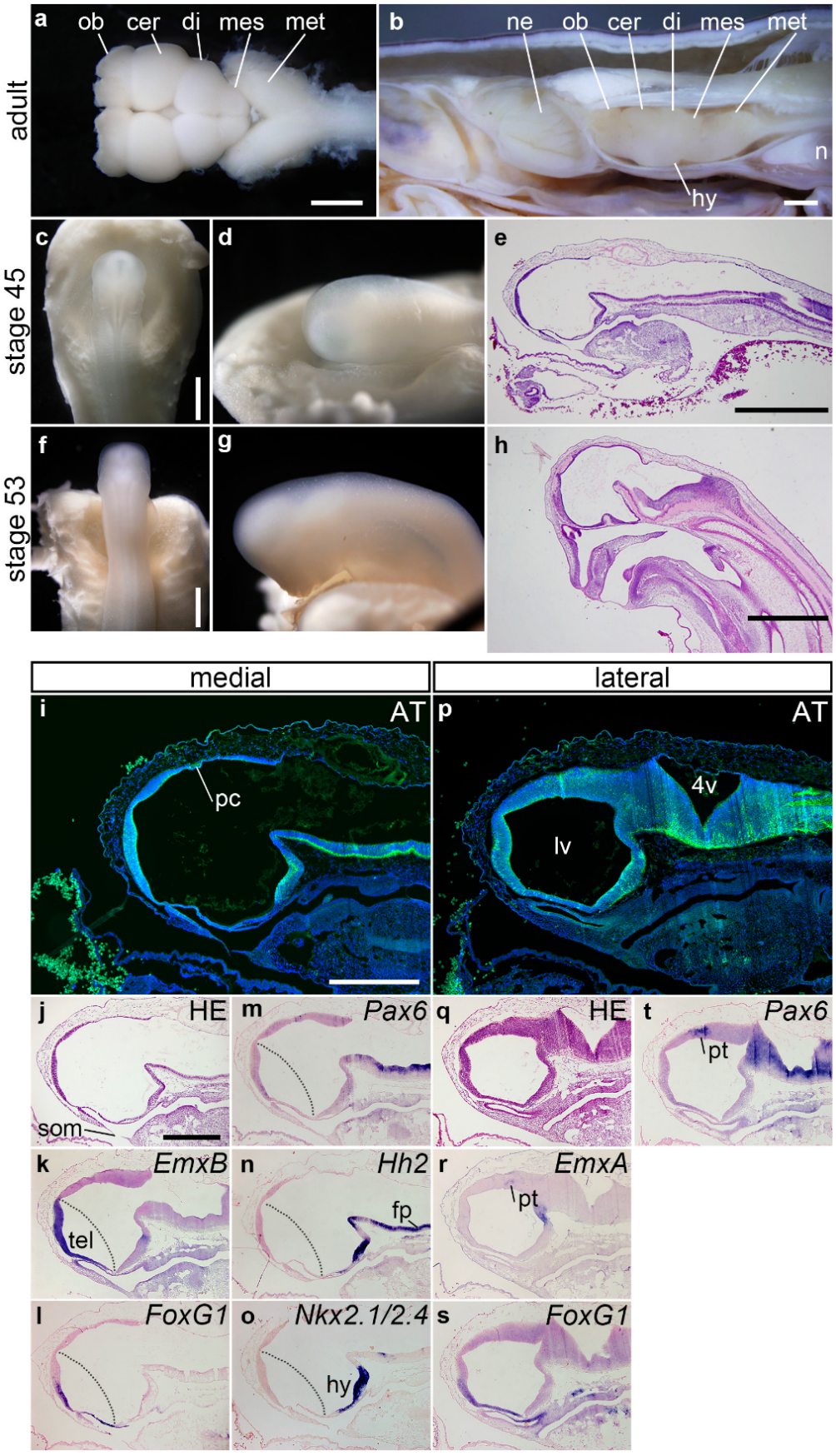
study. **j**, An evolutionary scenario of the vertebrate brain. Key: DP, dorsal pallium; fp, floor plate; VP, ventral pallium. See **Figs 1–3** for other abbreviations. Scale bars, 500 μm (**a–d**) and 100 μm (**e–h**).




525

526 **Extended Data Figure 1 | Embryonic brain of a shark.** Gene expression patterns and
527 nerve tracts in a stage 27[34] embryo of the cloudy catshark, *Scyliorhinus torazame*, to
528 show the conserved developmental pattern of the jawed vertebrate brain. Whole-mount
529 views (**a–e**), sagittal sections of the brain (**g–k**) and transverse sections at the
530 telencephalon level (**m–q**; the sectioned region is shown by the dotted line in **g**), stained
531 using probes for *FoxG1* (**a**, **g**, **m**), *Pax6* (**b**, **h**, **n**), *Dlx2* (**c**, **i**, **o**), *Nkx2.1* (**d**, **j**, **p**) and *Shh*
532 (**e**, **k**, **q**). **f**, **l**, Confocal images of the central and peripheral nervous systems visualized
533 by immunofluorescence using an anti-acetylated tubulin antibody (green) and DAPI
534 (blue). **r**, Schematic drawing of the shark telencephalon, showing conserved gene
535 expression patterns among jawed vertebrates. Some of these patterns were described
536 previously[7]. Key: op, olfactory placode; rp, Rathke's pouch. See **Figs 1** and **2** for other
537 abbreviations Scale bars, 500 μm.

538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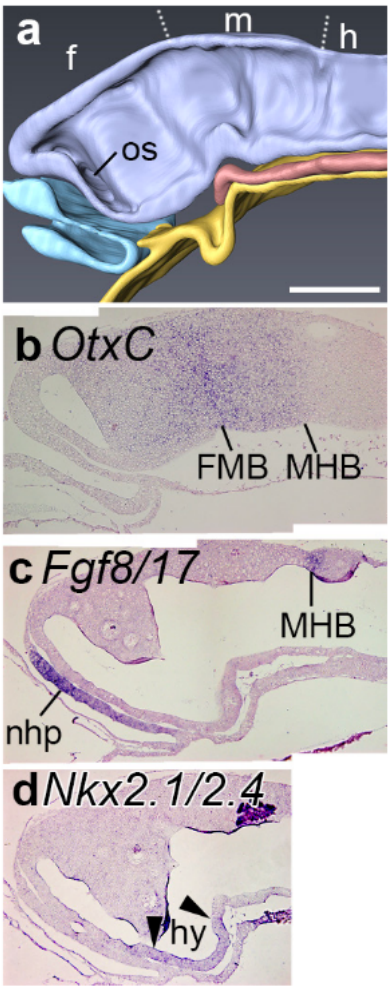
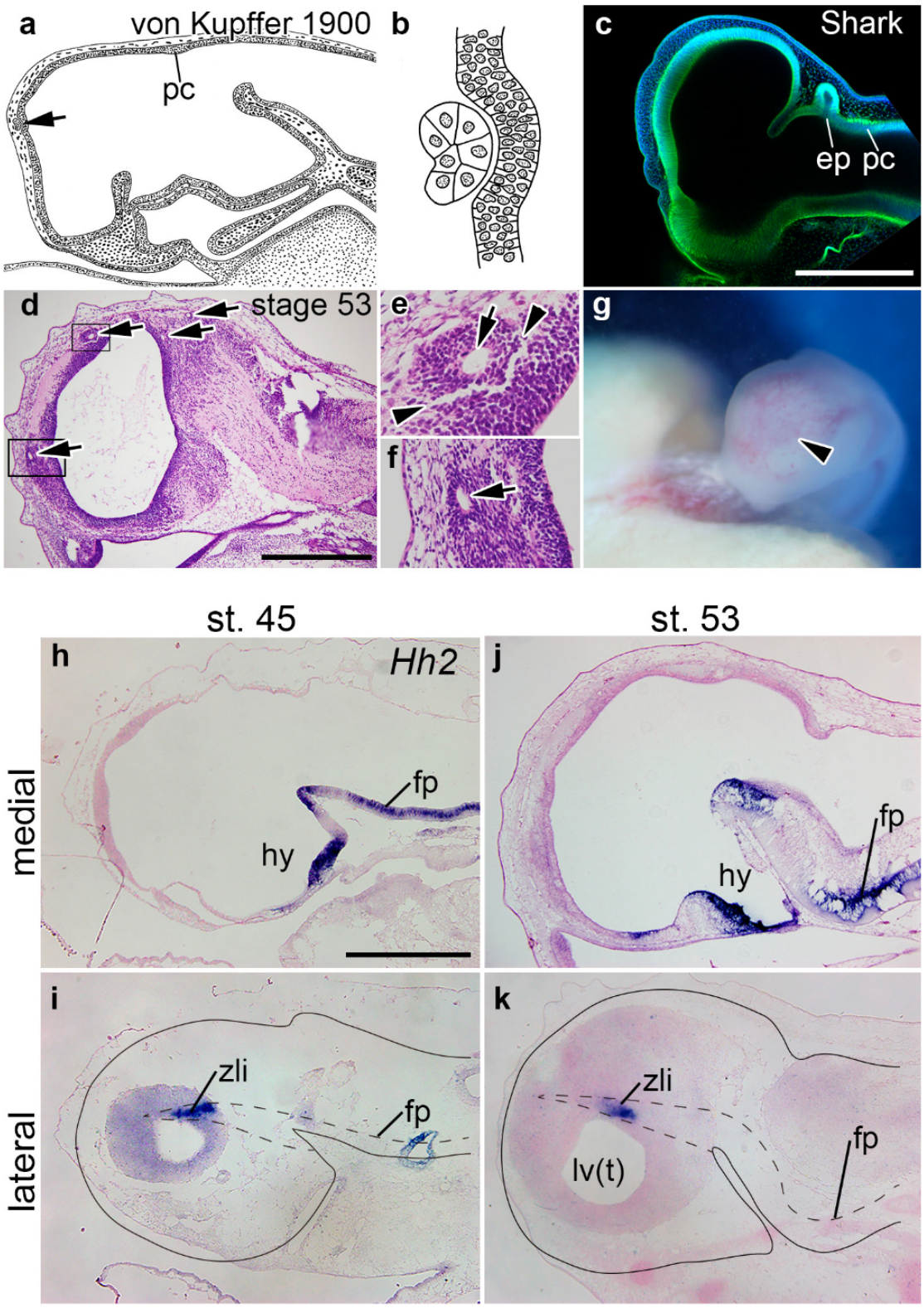
**Extended Data Figure 2 | Adult and embryonic brains of E. burgeri, and gene expression and nervous system staining of stage-45 embryo. a, b,** Dorsal view (**a**) and sagittal section (**b**) of the adult brain of *E. burgeri*. **c**–**h**, *E. burgeri* embryos at Bashford Dean stages 45 (**c**–**e**) and 53 (**f**–**h**)17,18. Dorsal (**c**, **f**) and lateral (**d**, **g**) views are shown. **e**, **h**, Sagittal sections stained with haematoxylin and eosin. Dean stage 53 is very similar to the embryo described in figure 1 in ref. 45. **i**–**t**, A stage 45 *E. burgeri* embryo observed using histological preparations. Dotted lines indicate the presumptive telencephalic border estimated by *EmxB* and *FoxG* expressions[21,22]. At this stage, *Nkx2.1/2.4* and *Hh2* have not been expressed yet in the rostral telencephalon (according to the prosomeric model[3]). cer, cerebrum; met, metencephalon; mes, mesencephalon; ob, olfactory bulb. See Figs 1, 2 and 4 for other abbreviations. Scale bars, 2.0 mm (**a**–**c** and **f**), 1.0 mm (**e**, **h**) and 500 μm (**i**, **j**).

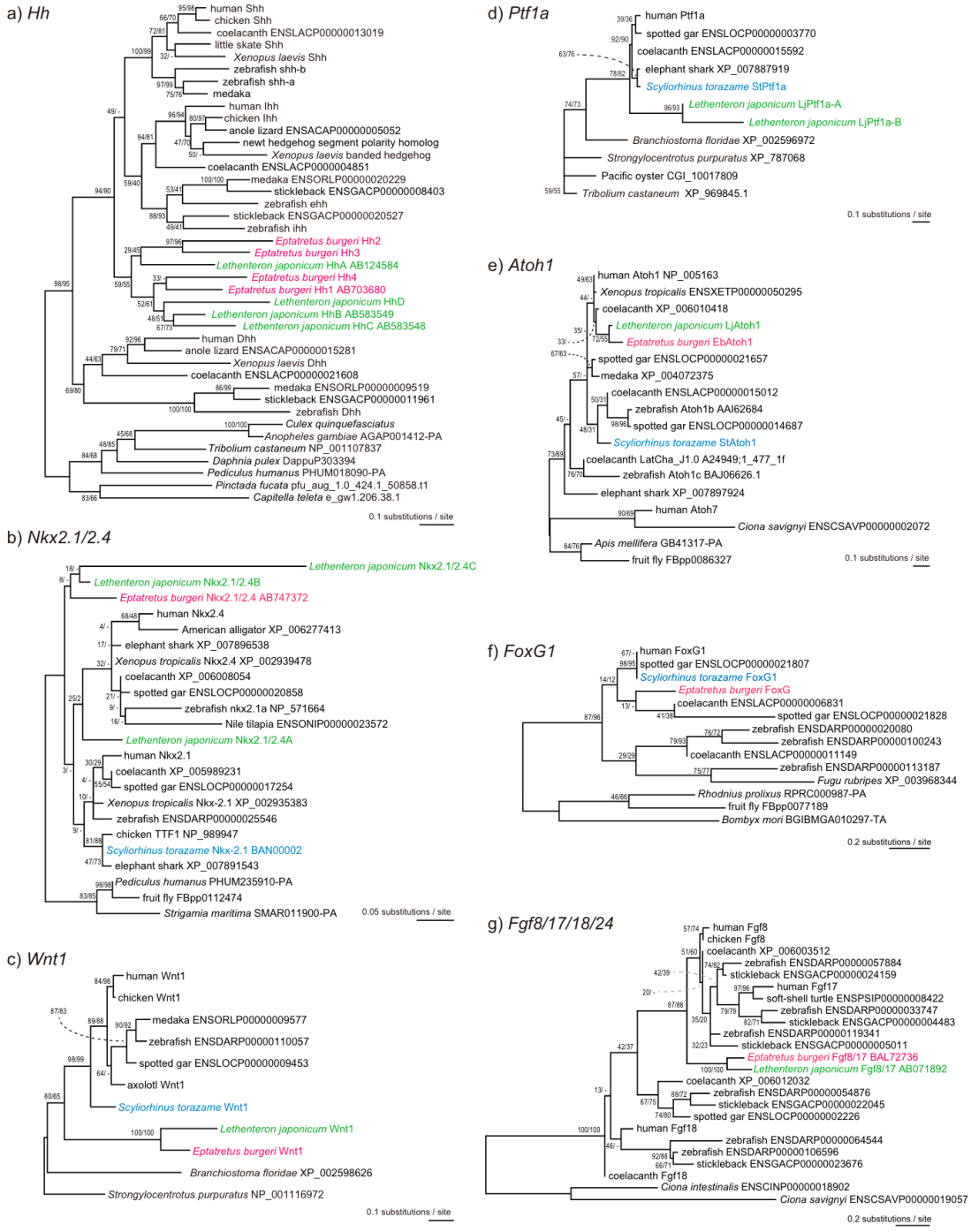



**Extended Data Figure 3 | Identification of the forebrain–midbrainhindbrain boundaries in early hagfish embryos. a–c,** The mid–hindbrain boundary (MHB) of the hagfish brain is assumed to correspond to the posterior expression boundary of *OtxC* (**b**) and focal expression of Fgf8/17 (see Extended Data Fig. 5) (**c**) that become evident at stages 28–30. **a**–**d**, On the other hand, the fore–midbrain boundary (FMB) is suggested morphologically (**a**, **b**) and by the ventrocaudal portion of *Nkx2.1/2.4* expression domain (**d**, arrowheads). See Figs 1 and 2 for abbreviations. Scale bar, 200 μm.



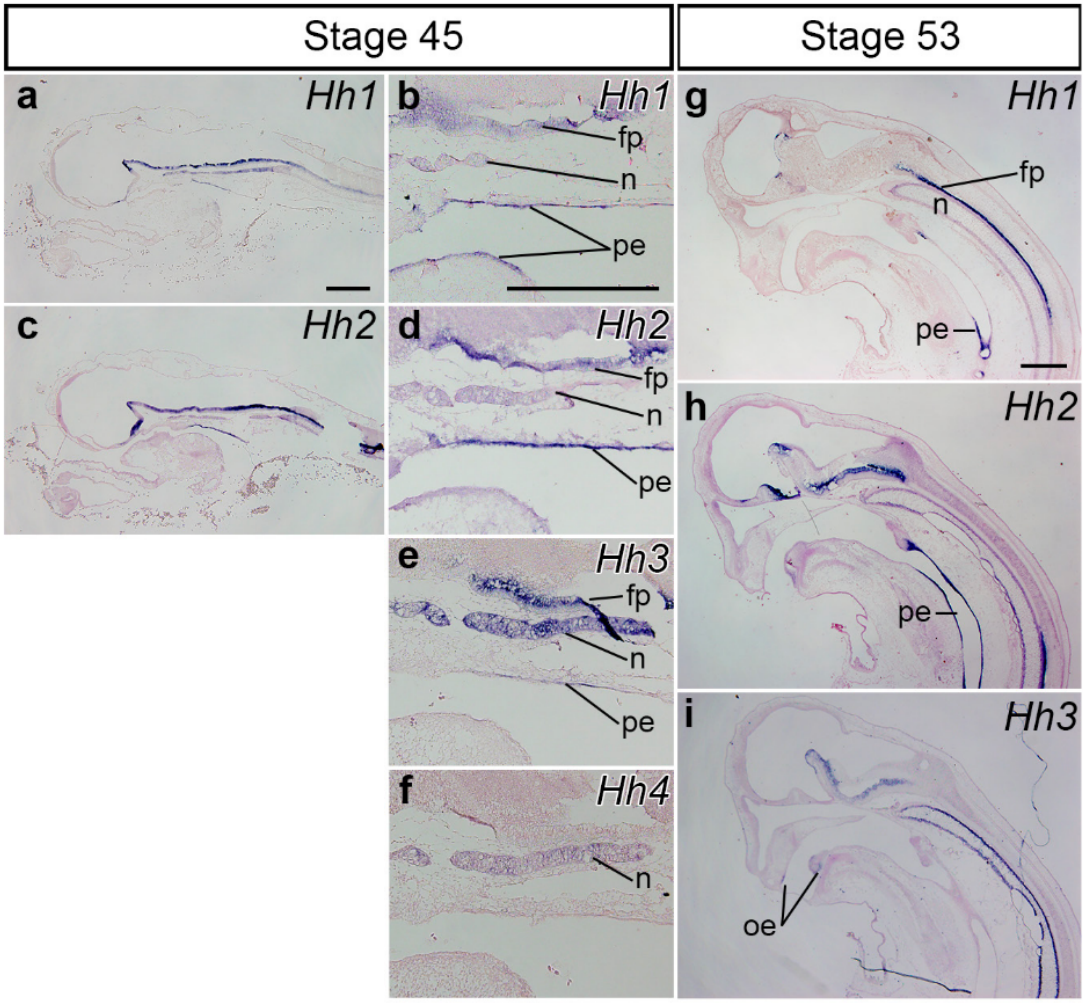

**Extended Data Figure 4 | Absence of the epiphysis and presence of the ZLI in the developing brain of the hagfish. a, b,** The adult hagfish has no epiphysis. A

rudimentary epiphysis described previously[14,46] was likely to have been an artefact derived from an inappropriate method of fixation. Although von Kupffer (1900) described an epiphysis-like structure in his illustration (**a**, **b**, redrawn from ref. 15), he denied its identity as an epiphysis because it is distantly positioned from the posterior commissure (pc). **c**, The position of the epiphysis (ep) in a shark embryo is shown. **d**–**g**, In our present observation of hagfish embryos also, several neuroepithelial cysts were found in the forebrain (arrows in **d**–**f)**, which were always associated with the development of blood vessel formation in the brain (arrowhead in **g**). Arrowheads in e indicate the crack between the neuroepithelial cyst and the brain tissue caused during the dehydration process, reminiscent of von Kupffer's epiphysis-like structure in **b**. **h**–**k**, Hh2 expression in stage-45 (**h**, **i**) and stage-53 (**j**, **k**) hagfish embryos. Medial sections (**h**, **j**) and lateral sections (**i**, **k**). Dotted lines in i and k indicate the presumptive ZLI region. See Figs 1–3 for abbreviations. Scale bars, 500 μm.




**Extended Data Figure 5 | Molecular phylogenetic trees of genes identified in this study.** The trees were inferred with the maximumlikelihood method using PhyML3.0 with the JTT+G4 model. Members of individual gene families were collected from public databases, and groupings of the sequences identified in this study into the subfamilies

shown in this figure were confirmed by preliminary phylogenetic inferences including other subfamilies. **a**, *Hedgehog* homologues (shape parameter for the gamma distribution alpha = 0.80). A total of 249 amino acid sites unambiguously aligned without any gap were used in the inference. **b**, *Nkx2.1/2.4* homologues (86 sites; alpha =0.60). **c**, Wnt1 homologues (196 sites; alpha =0.60). **d**, Ptf1a homologues (67 sites; alpha =0.92). **e**, *Atoh1* homologues (60 sites; alpha =0.74). **f**, *Fgf8/17/18/24* homologues including the hagfish homologue identified previously19 (74 amino acid sites; alpha =0.87). **g**, *FoxG1* homologues (115 amino acid sites; alpha = 0.16). Hagfish, lamprey and catshark sequences are shown in red, green and blue, respectively. Support values at nodes are bootstrap probabilities in the maximum-likelihood method and those in the neighbour-joining method (under the above-mentioned substitution model), in order.

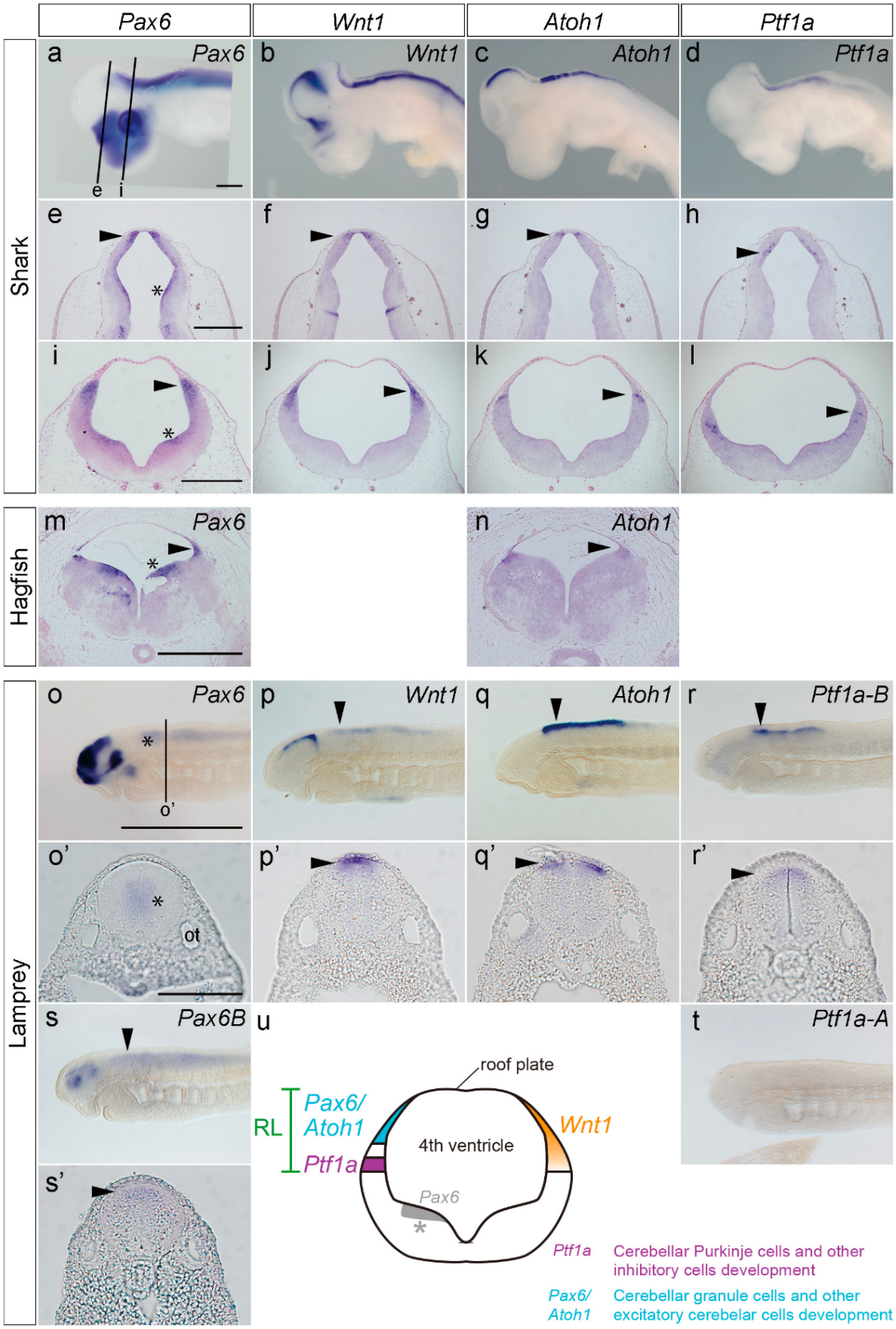



| j | *Hh1* | | *Hh2* | | *Hh3* | | *Hh4* | |
|---|---|---|---|---|---|---|---|---|
| | st. 45 | st. 53 | st. 45 | st. 53 | st. 45 | st. 53 | st. 45 | st. 53 |
| Telencephalon | − | − | − | + | nd | − | nd | nd |
| Hypothalamus | + | + | + | + | nd | − | nd | nd |
| *Zli* | nd | nd | + | + | nd | nd | nd | nd |
| Floor plate | + | + | + | + | + | + | − | nd |
| Notochord | + | − | + | + | + | + | + | nd |
| Pharyngeal endoderm | + | + | + | + | + | − | − | nd |
| Oral ectoderm | − | − | − | − | nd | + | nd | nd |

+, present; −, absent; nd, no data

**Extended Data Figure 6 | Expression patterns of hagfish Hedgehog genes. a–i,** In situ hybridization staining of four Hedgehog genes (*Hh1–4*) of *E. burgeri* at stage-45 (**a**–**f**) and stage-53 (**g**–**i**) embryos. The *Hedgehog* expressions were found in notochord

(n), floor plate (fp), pharyngeal endoderm (pe), oral ectoderm (oe) and subregions of the forebrain (hypothalamus, ZLI and MGE; see Fig. 3). The overall expressions of hagfish *Hh1–4* exhibited patterns similar to those of *Hedgehog* genes in jawed vertebrates, although each Hh expression showed slight differences in some domains. j, Summary of the expression patterns of *Hedgehog* genes (Hh1–4) of E. burgeri in stage 45 and stage 53 embryos. Scale bars, 500 μm.

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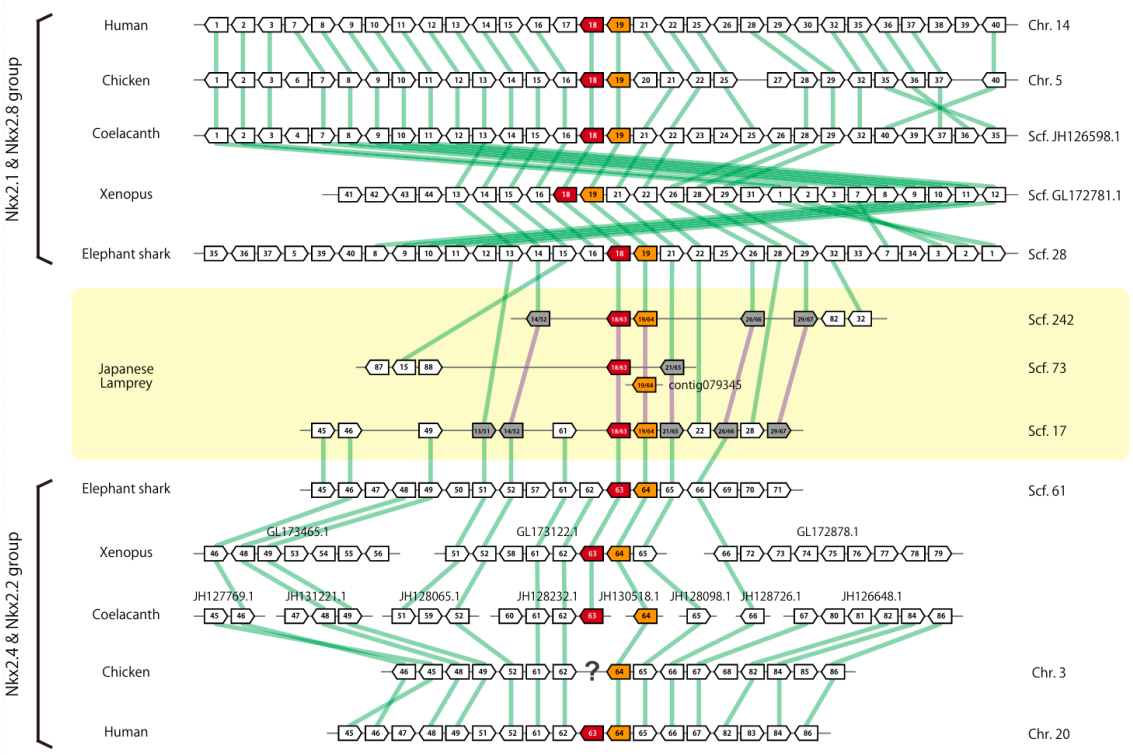
609 **Extended Data Figure 7 | Rhombic lip gene expression patterns in shark, hagfish**

**and lamprey embryos.** Gene expression patterns in stage-27 (whole-mount) and stage-31 (transverse sections) embryos of a catshark (S. torazame) (**a**–**l**), stage-53 embryo of a hagfish (*E. burgeri*) (**m**, **n**), and stage-26 embryo of lamprey (*L. japonicum*) (**o**–**t**), stained using probes for the *Pax6*, *Wnt1*, *Atoh1* and *Ptf1a* gene homologues. **u**, Schematic transverse section of the vertebrate rhombic lip showing crucial gene expression patterns based on ref. 27 and the present study. Lines in a indicate the levels of the transverse sections shown in **e**–**h** (rhombomere 1: the cerebellar primordia) and **i**–**l** (posterior rhombomeres). The line in **o** indicates the level of the transverse sections shown in (**o'**–**s'**) (around rhombomere 4). Arrowheads indicate rhombic lip gene expressions. Asterisks indicate non-rhombic lip expressions of *Pax6* through the neural tube. See Figs 1 and 2 for abbreviations. Scale bars, 500 μm (**a**, **e, i**, **m**, **o**) and 100 μm (**o'**).




623

624 **Extended Data Figure 8 | Synteny conservation between the genomic regions**

625 **containing *Nkx2.1/2.4* among gnathostomes and *L. japonicum*.** Numbered boxes

626 represent single protein-coding genes; all the genes used for synteny analysis and the

627 given numbers are listed in Extended Data Table 1. Colours of boxes are as follows: red,

628 *Nkx2-1/2-4* orthologue; orange, *Nkx2-2/2-8* orthologue; grey, a gene showing high

629 sequence similarity to two gnathostome ohnologues as a result of the TBLASTN search.

630 The green line represents orthology between the neighbouring vertebrate genomes,

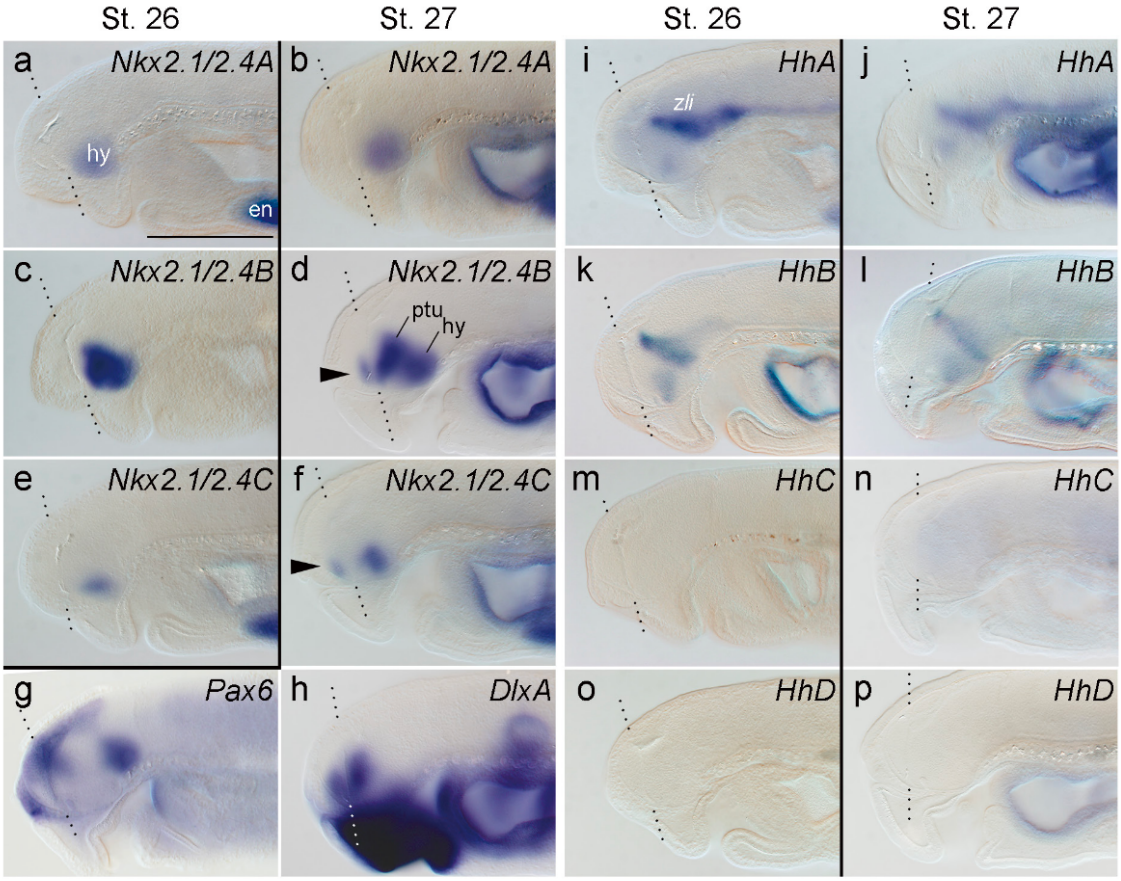
631 whereas the purple line indicates a paralogous relationship of each gene among the

632 lamprey genome scaffolds. At present, there are no descriptions of chicken NKX2-4

633 (nor in birds) and we have not been able to find it. This putative lack is marked by a

634 question mark.

635




q

| | *Nkx2.1/2.4A* | *Nkx2.1/2.4B* | *Nkx2.1/2.4C* |
|---|---|---|---|
| Telencephalon | – | + | + |
| Hypothalamus | + | + | + |
| Posterior tuberculum | – | + | – |
| Endostyle | + | + (weak) | + |

| | *HhA* | *HhB* | *HhC* | *HhD* |
|---|---|---|---|---|
| Telencephalon | – | – | – | – |
| Hypothalamus | + | + | – | – |
| *Zli* | + | + | – | – |
| Floor plate | + | + | – | – |
| Notochord | + | + | – | – |
| Pharyngeal endoderm | + | + | – | – |
| Oral ectoderm | – | – | – | – |
| Endostyle | + | – | – | – |

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**Extended Data Figure 9 | Telencephalic gene expression patterns in lamprey embryos. a–p**, Gene expression patterns in L. japonicum stage-26 and -27 (ref. 33) embryos, stained using probes for *Nkx2.1/2.4A* (**a**, **b**), *Nkx2.1/2.4B* (**c**, **d**), *Nkx2.1/2.4C* (**e**, **f**), *Pax6* (**g**), *DlxA* (**h**), *HhA* (**i**, **j**), *HhB* (**k**, **l**), *HhC* (**m**, **n**) and *HhD* (**o**, **p**). Dotted lines indicate the telencephalic border distinguished by the anterior intraencephalic sulcus[25]. The expression domains of *Pax6* (**g**) and *DlxA* (**h**) in the telencephalon pallial and subpallial subdivisions, respectively. Although the previously reported *Nkx2.1/2.4A* is not expressed in the telencephalon36, *Nkx2.1/2.4B* and *Nkx2.1/2.4C* are expressed in the rostral telencephalon (arrowheads), suggesting the presence of the MGE in the lamprey. We did not detect expression of any Hedgehog genes in the rostral telencephalon[25] (**i**–**p**). q, Summary of the expression patterns of *Nkx2.1/2.4* and *Hedgehog* genes of *L. japonicum* at embryonic stages 17–30 examined by whole-mount in situ hybridization. en, endostyle; ptu, posterior tuberculum47. See Figs 1 and 2 for other abbreviations. Scale bar, 200 μm.

**Extended Data Table 1 | List of genes used in the synteny conservation analysis.**

Each gene number corresponds to the numbers in **Extended Fig. 13**.

| *Nkx2.1* & *Nkx2.8* group | | *Nkx2.4* & *Nkx2.2* group | |
|---|---|---|---|
| Number | Gene name | Number | Gene name |
| 1 | SNX6 | 45 | SLC24A3 |
| 2 | CFL2 | 46 | RIN2 |
| 3 | BAZ1A | 47 | NAA20 |
| 4 | ENSLACG00000017921 | 48 | CRNKL1 |
| 5 | SINCAMG00000016188 | 49 | CFAP61 |
| 6 | ENSGALG00000010034 | 50 | GTF2IRD2 |
| 7 | SRP54 | 51 | INSM1 |
| 8 | FAM177A1 | 52 | RALGAPA2 |
| 9 | PPP2R3C | 53 | ATP2A1 |
| 10 | KIAA0391 | 54 | ENSXETG00000032786 |
| 11 | PSMA6 | 55 | TCTN3 |
| 12 | NFKBIA | 56 | PPP1CA |
| 13 | INSM2 | 57 | SINCAMG00000008965 |
| 14 | RALGAPA1 | 58 | ENSXETG00000009758 |
| 15 | BRMS1L | 59 | ENSLACG00000009798 |
| 16 | MBIP | 60 | ENSLACG00000001348 |
| 17 | SFTA3 | 61 | KIZ |

| | | | |
|---|---|---|---|
| 18 | NKX2.1 | 62 | XRN2 |
| 19 | NKX2.8 | 63 | NKX2.4 |
| 20 | ENSGALG00000010113 | 64 | NKX2.2 |
| 21 | PAX9 | 65 | PAX1 |
| 22 | SLC25A21 | 66 | FOXA2 |
| 23 | ENSLACG00000014546 | 67 | SSTR4 |
| 24 | ENSLACG00000014339 | 68 | THBD |
| 25 | MIPOL1 | 69 | SINCAMG00000009008 |
| 26 | FOXA1 | 70 | SFT2D1 |
| 27 | ENSGALG00000010121 | 71 | PRR18 |
| 28 | TTC6 | 72 | CFAP36 |
| 29 | SSTR1 | 73 | SMEK2 |
| 30 | CLEC14A | 74 | PNPT1 |
| 31 | CAPN3 | 75 | EFEMP1 |
| 32 | SEC23A | 76 | CCDC85A |
| 33 | HRH2 | 77 | VRK2 |
| 34 | SINCAMG00000016299 | 78 | FANCL |
| 35 | GEMIN2 | 79 | ENSXETG00000020482 |
| 36 | TRAPPC6B | 80 | ENSLACG00000017923 |
| 37 | PNN | 81 | ENSLACG00000017898 |
| 38 | MIA2 | 82 | CD93 |
| 39 | CTAGE5 | 83 | NXT1 |
| 40 | FBXO33 | 84 | GZF1 |

| | | | |
|---|---|---|---|
| 41 | CHST9 | 85 | ENSGALG00000008350 |
| 42 | ITPKA | 86 | NAPB |
| 43 | ZNF106 | 87 | NUDT18 |
| 44 | EAPP | 88 | PRDX5 |

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