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Fgf8 gene regulatory network and the isthmic organizer: an evolutionary perspective

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ABSTRACT The midbrain-hindbrain boundary (MHB), also known as the isthmic organizer (IsO), plays a critical role in the developmental patterning of the posterior midbrain and anterior hindbrain. Understanding the wiring of this organizer's deeply conserved gene regulatory network is of significant interest for both evolutionary and neurodevelopmental biology. Various secreted signalling molecules and transcription factors have been identified as being important components for the formation and function of the MHB. Among these, FGF8 is considered a primary mediator of IsO activity; it directs anterior-posterior patterning and promotes the specification and maintenance of the MHB. While the core gene regulatory network governing MHB development is well-characterized, the direct interactions between key regulatory genes and the cis-regulatory elements that control their spatiotemporal expression remain poorly understood. This review summarizes the current knowledge of the gene regulatory network underlying the formation of the vertebrate midbrain-hindbrain boundary. We focus in particular on Fgf8 and its regulatory landscape from an evolutionary perspective.

KEYWORDS: Fgf8, midbrain-hindbrain boundary, gene regulatory networks, enhancer, regulatory landscape

Introduction

The vertebrate brain is a highly complex organ that develops from a simple sheet of epithelial cells known as the neural plate. During development, this initially simple structure becomes progressively more complex as the cells of the embryo divide, migrate, and acquire specific developmental identities. Neurulation transforms the neural plate into the neural tube through a multistep process that involves morphogenetic and inductive processes in which the brain is formed anteriorly and the spinal cord posteriorly. Subsequently, the brain undergoes division into three primary vesicles: the forebrain, midbrain, and hindbrain. Later in development the forebrain and hindbrain subdivide into secondary vesicles that eventually give rise to the adult brain structures.

The patterning underlying this spatial organization of the brain is directed by specialized signalling centres, so called organizers, which via morphogenetic signalling can pattern and induce cell fates in adjacent cells. The tripartite division of the brain can be traced back to ancestral chordates during evolution (Wada *et al.*, 1998) and several of the secondary organizers coordinating vertebrate brain patterning have been proposed to have ancient deuterostome origins (Imai *et al.*, 2009; Pani *et al.*, 2012). Among them is the midbrain-hindbrain boundary (MHB), or isthmic organizer (IsO), which is essential for the developmental patterning of the posterior midbrain and anterior hindbrain (Wurst and Bally-Cuif, 2001). The deeply conserved MHB gene regulatory network (GRN) (Pani *et al.*, 2012) makes its study interesting from an evolutionary perspective but it is also important to understand the underlying developmental mechanisms that can be disturbed in congenital disorders of the midbrain and hindbrain (Doherty *et al.*, 2013; Gibbs *et al.*, 2017).

Various secreted signalling molecules and transcription factors have been identified in the gene regulatory network that direct MHB specification and maintenance. Among them are Fibroblast growth factor 8 (*Fgf8*), members of the wnt-family of proteins (*Wnt1*, *Wnt3*, *Wnt10b*), as well as *Paired homeobox 2/5/8* (*Pax*2/5/8), Engrailed 1/2 (*En1/2*) and *Lim homeobox 1b (Lmx1b)* transcription factors (Hidalgo-Sánchez *et al.*, 2022; Wurst and Bally-Cuif, 2001). Several lines of evidence suggest that the main inductive molecule mediating IsO activity and directing anterior-posterior patterning in this region is FGF8. It is expressed in the MHB in all major vertebrate lineages (Christen and Slack, 1997; Crossley and Martin, 1995; Hidalgo-Sánchez *et al.*, 1999a; Reifers *et al.*, 1998). Furthermore, both loss-of-function experiments and ectopic expression experiments have demonstrated that it plays an essential role in the induction and maintenance of this region (Chi *et al.*, 2003; Crossley *et al.*, 1996; Irving and Mason, 2000;

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Jászai *et al.*, 2003; Liu *et al.*, 1999; Martinez *et al.*, 1999; Meyers *et al.*, 1998; Reifers *et al.*, 1998; Sato and Joyner, 2009). Even though the core gene regulatory network directing MHB development is well characterized, and the essential role of *Fgf8* in this system is well known, the direct interactions between key genes and their trans-acting regulatory factors are not well understood, nor is the evolution of the cis-regulatory elements that mediate their interactions. We will here review current knowledge on the MHB gene regulatory network with an emphasis on *Fgf8* and its regulatory landscape from an evolutionary perspective.

Positioning, activation and maintenance of the isthmic organizer in vertebrates

MHB formation involves multiple steps, including its positioning during gastrulation at the junction of *Otx* and *Gbx* expression, the establishment of the isthmus organizer activity through *Fgf8* expression, and the maintenance of its regional identity and organizing activities (Gibbs *et al.*, 2017; Wurst and Bally-Cuif, 2001). The development of the IsO is initiated within the neural plate as *Otx* and *Gbx* transcription factors are expressed. *Otx2* expression is localised to the prospective forebrain and midbrain, and is required for specification of the most anterior parts of the brain including the secondary prosencephalon, diencephalon and the mesencephalon (Rhinn *et al.*, 1998). *Gbx2,* on the other hand is expressed in the prospective hindbrain and is required for proper hindbrain development (Wassarman *et al.*, 1997). The *Gbx2* and *Otx2* expression domains are slightly overlapping before a sharp boundary becomes evident; at the onset of *Fgf8* MHB expression during early somitogenesis, these expression domains are adjacent but mutually exclusive (Fig. 1). The juxtaposition of *Otx* and *Gbx* gene expression is evolutionary conserved in vertebrates and is important for the positioning of the IsO in *Xenopus* (Glavic *et al.*, 2002; Tour *et al.*, 2002a; Tour *et al.*, 2002b), zebrafish (Foucher *et al.*, 2006; Mercier *et al.*, 1995; Rhinn *et al.*, 2003; Rhinn *et al.*, 2009), chick (Garda *et al.*, 2001; Katahira *et al.*, 2000), and mouse (Broccoli *et al.*, 1999; Li and Joyner, 2001; Martinez-Barbera *et al.*, 2001; Millet *et al.*, 1999). Other IsO-core genes also define the expression boundary of *Otx* and *Gbx*, suggesting a complex and dynamic process of MHB establishment (Martinez-Barbera *et al.*, 2001; Rhinn *et al.*, 2003). Still, genetic ablation of *Gbx2* or *Otx2* in mouse (Li and Joyner, 2001; Millet *et al.*, 1999) and *Gbx1/2* in zebrafish (Su *et al.*, 2014) demonstrate that they are not required for the induction of *Fgf8* expression. Thus, although not properly positioned, the MHB gene expression programme is initiated even in the absence of these transcription factors. Once the IsO is established and *Fgf8* expression is induced, it requires the reciprocal repressive interaction of Otx2 and Gbx2 to be maintained (Li and Joyner, 2001; Rhinn *et al.*, 2003; Su *et al.*, 2014) (Fig. 1).

It has been proposed that several independent parallel signalling pathways are activated during the initial establishment of the MHB and that they involve *Pax2*, *Wnt1*, and *Fgf8* respectively (Rhinn and Brand, 2001). However, bead implantation experiments and genetic ablation have shown that *Fgf8* is the main inducer of posterior midbrain morphogenesis and anterior hindbrain patterning (Crossley *et al.*, 1996; Meyers *et al.*, 1998; Reifers *et al.*, 1998; Shamim *et al.*, 1999). Fgf8 ultimately induces tectum on its anterior side and cerebellum on its posterior side, while repressing the anterior-most hoxa2 expression in the anterior hindbrain (Irving and Mason, 2000; Sato and Joyner, 2009). The expression of *Fgf8* is initiated at the border between the *Otx* and *Gbx* domains and localized to the anterior hindbrain region overlapping with the anterior most Gbx2 expression (Hidalgo-Sánchez *et al.*, 1999b; Katahira *et al.*, 2000). Despite the essential importance of *Fgf8* expression in the IsO, no specific evolutionary conserved transcription factor has unambiguously been demonstrated to control induction of *Fgf8* expression in the IsO directly.

In mice, genetic perturbation experiments suggested that the LIM homeobox transcription factor 1 beta (Lmx1b) and the paired-box family (Pax2/5/8) of transcription factors were important for initiation of *Fgf8* expression (Guo *et al.*, 2007; Ye *et al.*, 2001). In mice, *Lmx1b* expression partially overlaps that of *Fgf8* during initiation of the IsO. Analysis of *Lmx1b*$^{-/-}$ mice suggested the complete absence of *Fgf8* expression in the MHB (Guo *et al.*, 2007). However, recent data using radioactive *in situ* hybridisation demonstrate that *Fgf8* expression is indeed induced in the MHB of these mice (Sherf *et al.*, 2015). Also, double knockdown of *Lmx1b.1* and *Lmx1b.2* in zebrafish lead to progressive loss of *Fgf8a* only during the maintenance phase (O'Hara *et al.*, 2005). Misexpression of *Lmx1b* in chick shows that *Fgf8* is only induced in the adjacent surrounding cells (Matsunaga *et al.*, 2002), further suggesting that any inductive effect of LMX1B on *Fgf8* expression is only indirect.

Although *Fgf8* MHB expression and associated anatomical structures are completely absent in *Pax2* knockout mice in the C3H/He genetic background, MHB specification and development is normal in the C57Bl/6 background (Schwarz *et al.*, 1997; Ye *et al.*, 2001). Also, in zebrafish *noi* mutants that carry a functional deletion of *Pax2a*, expression of the core MHB genes is initiated and then progressively lost in the maintenance phase (Lun and Brand, 1998). Despite these discrepancies, Pax transcription factors could potentially be important inducers of *Fgf8* gene expression since redundant function of other Pax transcription factor

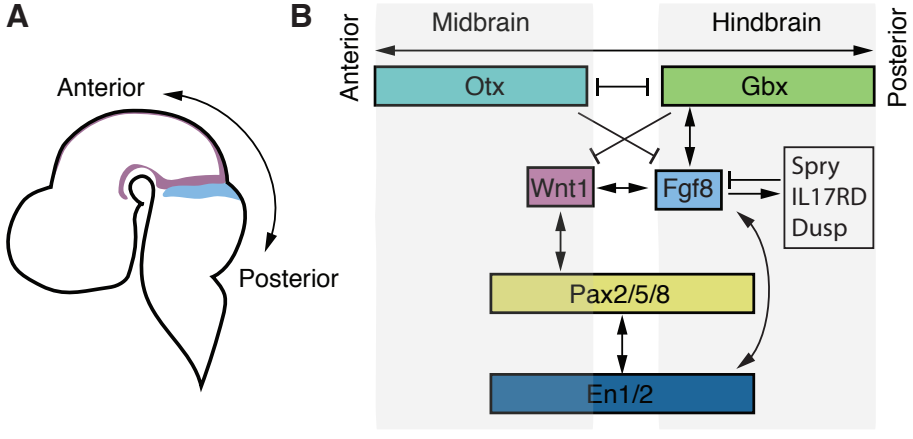

Fig. 1. Genetic interactions in the midbrain-hindbrain boundary (MHB). (A) Schematic drawing indicating the anatomical context shown in (B). **(B)** Illustration of key genetic interactions in the MHB region.

family members has been described (Schwarz *et al.*, 1997) that in combination with genetic variation could buffer for induction of *Fgf8* expression. Likewise, cooperativity and redundant function of distinct classes of trans-acting factors could potentially provide additional robustness during the induction of gene expression in the initiation phase. To further clarify this and identify direct regulators of *Fgf8*, genetic studies of compound mutants in combination with biochemical approaches are required.

Overall, previous studies have demonstrated an evolutionary conserved role in MHB development for several secreted molecules and transcription factors (Hidalgo-Sánchez *et al.*, 2022; Wurst and Bally-Cuif, 2001). The induction of gene expression for these core factors is dependent on parallel signalling pathways (Canning *et al.*, 2007; Lun and Brand, 1998) whereas the maintenance is characterized by extensive interdependent feedback loops (Dworkin and Jane, 2013; Gibbs *et al.*, 2017) (Fig. 1). In most cases, perturbation of these core factors does not affect the initial establishment of the MHB but still lead to severe defects in the maintenance of the IsO and to later malformations in the midbrain-hindbrain region (McMahon *et al.*, 1992; Lee *et al.*, 1997; Adams *et al.*, 2000; Lun *et al.*, 1998; Hirata, 2001; Itoh *et al.*, 2002; Buckles *et al.*, 2004; Chung *et al.*, 2006; Sherf *et al.*, 2015). This observation suggests that distinct regulatory cues direct the initiation and maintenance of IsO gene expression and that its patterning activity relies on the interdependent expression of these core MHB factors, mediated via positive and negative regulatory feedback loops (Hidalgo-Sánchez *et al.*, 2022; Wurst and Bally-Cuif, 2001) (Fig. 1). Thus, genetic studies in various vertebrate model organisms have led to a model of MHB development that includes positioning, initiation, and maintenance, and that involves a highly conserved core gene regulatory network but for which the initial inductive molecular cascade and direct regulatory events remain unknown.

Evolution of the midbrain-hindbrain boundary

During early development, the vertebrate brain is divided into three regions that give rise to the forebrain, the midbrain and the hindbrain. This tripartite regional organisation of the brain is thought to have ancient origin, predating the evolution of the chordate lineage (Lowe *et al.*, 2003; Wada *et al.*, 1998) and has been suggested to have been present in the last common urbilaterian ancestor (Hirth *et al.*, 2003; Urbach, 2007). Although there is a partial overlap between genes demarcating the tripartite subdivision of the brain, and those in the vertebrate MHB gene regulatory network, the origin and evolution of the MHB as an organizer is less well defined (Holland, 2015).

In *Drosophila melanogaster*, *Otx* and *Gbx* orthologues are expressed in a juxtaposed pattern similar to that of vertebrates. The boundary between the anteriorly expressed *Otd* (*Otx*) and the more posteriorly expressed *Unpg* (*Gbx*) aligns with the deutocerebral–tritocerebral boundary (DTB), which has been hypothesized to correspond to the vertebrate MHB (Bridi *et al.*, 2020; Hirth *et al.*, 2003; Urbach, 2007). Similar to vertebrates, the *D. melanogaster* orthologues of *Wnt1* and *En* are expressed in the vicinity of the *Otd-Unpg* interface. Initial reports describing the expression of *Fgf8*-orthologues did not support a role in *Drosophila* boundary formation (Hirth *et al.*, 2003; Urbach, 2007), but more recent data show that the *Fgf8*-orthologues *Ths* and *Pyr* are expressed in the DTB. Genetic experiments indicate that downregulation of these genes or the FGF8-receptor *Htl* leads to altered expression of *En* and *Unpg* (Bridi *et al.*, 2020). Although this suggests that FGF8 has an ancient role associated with boundary formation in the tripartite brain, it does not appear to have the organizing activity intrinsic to the vertebrate IsO (Bridi *et al.*, 2020).

In the hemichordate *S. kowalevskii*, a gene expression programme reminiscent of that in the vertebrate MHB is present in the developing ectoderm (Lowe *et al.*, 2003; Pani *et al.*, 2012). Importantly, the juxtaposed expression pattern of homologues to *Wnt1* and the key MHB inducer *Fgf8* (*Fgf8/17/18*) is localized to adjacent domains at the collar–trunk coelom boundary, respectively (Fig. 2). Similar juxtaposition is also evident in the case of the transcription factors *Otx* and *Gbx*, while *Pax2/5/8* and *En* are also expressed in the region (Fig. 2). However, *Otx-Gbx* expression is reversed as compared to *Fgf8-Wnt1* expression in *S. kowalevskii*: *Otx* expression overlap with

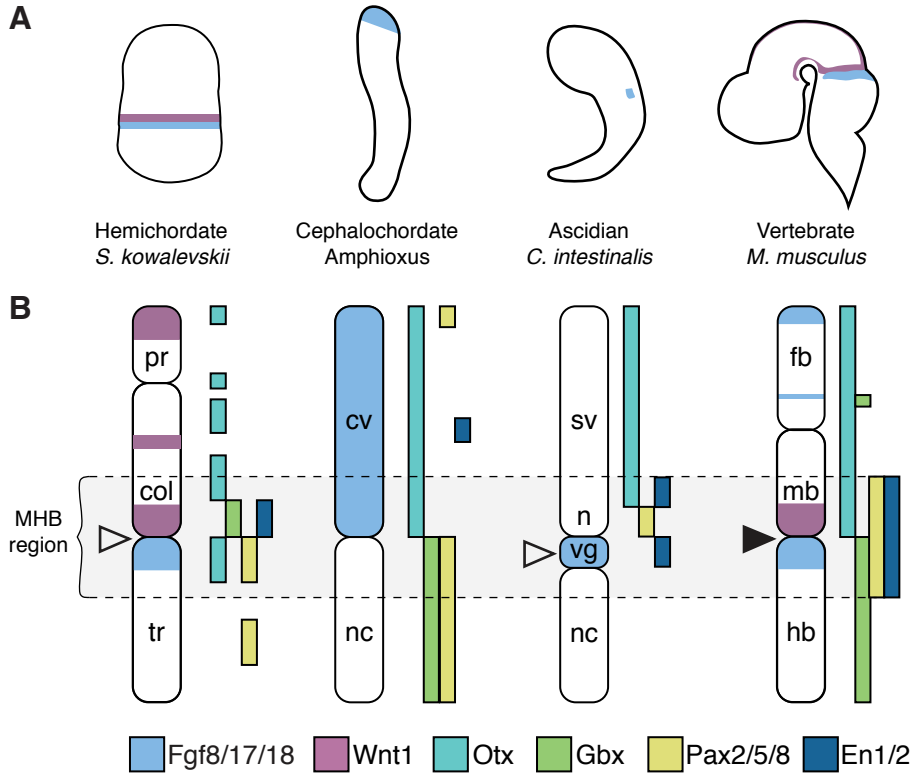

Fig. 2. Chordate expression of midbrain-hindbrain boundary (MHB) genes. (A) The anatomical context shown in (B). **(B)** A summary of the gene expression patterns of *Fgf8*, *Wnt1*, *Otx*, *Gbx*, *Pax* and *En* homologues in the ectoderm of ***S. kowalevskii***, amphioxus, and *C. intestinalis*, and in the brain of *M. musculus*. col: collar, cv: cerebral vesicle, fb: forebrain, hb: hindbrain, mb: midbrain, n: neck, nc: nerve cord, pr: proboscis, sv: sensory vesicle, tr: trunk, vg, visceral ganglion. White arrowheads indicate the presumptive IsO in *S. kowalevskii* and *C. intestinalis*; the black arrowhead indicates the vertebrate IsO. The diagrams are not drawn to scale.

that of *Fgf8* while *Gbx* expression overlap with that of *Wnt1*. Also distinct to vertebrates, the *Pax2/5/8* and *En* expression domains do not overlap. Still, based on these data, and those from experiments in which FGF and WNT signalling was perturbed, it has been suggested that the gene regulatory network directing the MHB secondary organizer has ancient deuterostome origin pre-dating vertebrate evolution (Pani *et al.*, 2012).

In the cephalochordate amphioxus, *Otx* and *Gbx* genes are expressed in juxtaposition at the border between the cerebral vesicle and the hindbrain in a region corresponding to the MHB (Castro *et al.*, 2006). However, other genes involved in vertebrate IsO specification display distinct expression patterns: *Fgf8/17/18* is expressed in the entire cerebral vesicle and *Wnt1* is not expressed in the region (Bertrand *et al.*, 2011; Holland, 2013), while *Pax2/5/8* is expressed throughout the hindbrain (Holland, 2013; Kozmik *et al.*, 1999) (Fig. 2). This suggests that amphioxus have some of the genetic machinery required for specification of the IsO but that the key molecules mediating organizing activity in the vertebrate IsO are lacking and their cross-regulatory interactions are not present (Holland, 2015).

Data from the urochordate *C. intestinalis* show that expression of the *Fgf8/17/18* orthologue is localized to the brain larval visceral ganglion at the tailbud stage, juxtaposed to *Pax2/5/8* expression and (as it is in the vertebrate IsO) co-expressed with *En* (Imai *et al.*, 2002; Imai *et al.*, 2009) (Fig. 2). Knockdown of *Fgf8/17/18* leads to loss of *Pax2/5/8* expression and expansion of *Otx* and *FoxB*, which are both expressed in the anterior central nervous system domain, adjacent to *Pax2/5/8* (Imai *et al.*, 2009). This change resembles the transformation of the anterior hindbrain into an expanded midbrain that occurs in vertebrates in *Fgf8* loss of function mutants (Chi *et al.*, 2003; Reifers *et al.*, 1998). Thus, although it has been suggested that this *Fgf8/17/18* expression could also correspond to the more posterior expression in rhombomere 4 in zebrafish (Cañestro *et al.*, 2005), it appears that *Fgf8/17/18* in *C. intestinalis* mediates organizing activity that is important for midbrain-hindbrain regionalisation, which in turn suggests that it corresponds at least to a partial IsO homologous to that in the vertebrate MHB (Imai *et al.*, 2009).

Still, in vertebrates, the positioning and maintenance of the *Fgf8* expression in the IsO depends on the combined cross-regulatory interactions of *Wnt1*, *Otx, Gbx, Pax2/5/8, En1/2,* among others. While *En* expression overlap with *Fgf8/17/18* in *C. intestinalis*, the expression domain of *Pax2/5/8* is located more anteriorly, and *Wnt1* and *Gbx* are not present in the *C. intestinalis* genome (Dehal *et al.*, 2002; Hino *et al.*, 2002; Wada *et al.*, 2003) (Fig. 2). Furthermore, in contrast to vertebrates but similar to the regulation of the *Drosophila Fgf8* homologues *Ths* and *Pyr* (Stathopoulos *et al.*, 2004), the expression of *C. intestinalis Fgf8/17/18* is restricted by the transcriptional repressor *Snail* (Imai *et al.*, 2009). These studies indicate that while some of the genetic components of the IsO existed before chordate evolution, the complete cross-regulatory interactions that induce and maintain the vertebrate IsO likely evolved after the divergence of the cephalochordates and olfactores lineages. While the GRN that patterns the MHB appears to have an ancient origin, it is not yet fully established to what extent the neural circuits arising from this region in invertebrates perform functions comparable to those in vertebrates. Still, in *D. melanogaster*, the brain region derived from the DTB integrates sensorimotor information and mediates balance and motor coordination. This may imply that the vertebrate MHB originated from an ancestral region already characterized by the presence of neural circuits related to balance and vestibular function (Bridi *et al.*, 2020). Additionally, co-expression of *En* and *tyrosine hydroxylase* in the *S. kowalevskii* collar-trunk coelom boundary indicates that these cells may be homologous to vertebrate midbrain dopaminergic neurons.

In none of the species that display IsO-resembling *Fgf8 gene* expression, have the upstream transcription factors and the regulatory events that lead to its initiation been reported, and it remains unclear how the regulation of *Fgf8* evolved. Nevertheless, despite variations in the genetic networks specifying the MHB, the findings from these evolutionarily distant species suggest that *Fgf8* had an ancestral role in boundary formation that later evolved to include the organizing activity that is crucial for the vertebrate IsO.

The FGF protein family and signalling

The Fibroblast Growth Factor (FGF) protein family is a group of structurally related growth factors that are crucial in numerous biological processes. During embryonic development they are important for cell proliferation, differentiation, migration, and survival in multiple organs, making them essential for tissue patterning and morphogenesis. In the MHB region, in addition to induction and maintenance of the MHB GRN, FGF-signalling have multiple functions, including cell survival (Basson *et al.*, 2008), maintenance of symmetrical proliferative divisions in the midbrain ventricular zone (Lahti *et al.*, 2011), and axonal guidance (Irving *et al.*, 2002). Based on phylogenetic analyses in humans and mice, 22 FGF members are divided into seven subfamilies — Fgf1, Fgf4, Fgf7, Fgf8, Fgf9, Fgf11, and Fgf15/19 (Ornitz and Itoh, 2022). These subfamilies are further classified into three functional groups: canonical, hormone-like, and intracellular FGFs. Canonical FGFs, which include the FGF8 as well as the FGF1, FGF4, FGF7, and FGF9 subfamilies, are primarily secreted ligands that bind tightly to heparan sulphate proteoglycans, which regulate their interactions with specific FGF receptors (Mohammadi *et al.*, 2005; Yayon *et al.*, 1991).

In zebrafish, the genome contains 27 *Fgf* genes, with all seven subfamilies represented and two paralogues for some genes (including *Fgf8*) due to the genome duplication that occurred after the teleost split (Itoh and Konishi, 2007). The expansion of FGF genes likely occurred in two major phases, first after the separation of the protostome and deuterostome lineages and then a second expansion in early vertebrate evolution (Itoh and Ornitz, 2011). Reflecting this time frame, the *C. intestinalis*, amphioxus and *S. kowalevskii* genomes harbour 6, 8 and 6 *Fgf* genes, respectively (Bertrand *et al.*, 2011; Oulion *et al.*, 2012; Satou *et al.*, 2002), while *D. melanogaster* and the nematode *C. elegans* have 3 FGF genes. Members of the FGF8 sub-family are present in all of these species and have been described in various arthropods (Oulion *et al.*, 2012).

FGF-signalling is mediated by four distinct high-affinity receptor tyrosine kinases in vertebrates, originating from one single gene in primitive chordates. Although FGFR1, FGFR2, and FGFR3 are all expressed in the embryonic midbrain in mice, only FGFR1 expression overlaps with that of Fgf8 at the IsO (Blak *et al.*, 2005), and mice lacking FGFR1 expression manifest a more severe MHB phenotype than mice lacking FGFR2 or FGFR3 (Blak *et al.*, 2007; Trokovic *et al.*, 2003). Still, FGFR2 and FGFR3 are also thought to be important transducers of the IsO signal, and act redundantly in conjunction with FGFR1 to regulate the production of neuronal cells such as dopaminergic neurons in the ventral midbrain, as well

as promoting survival of dorsal neuroectoderm (Saarimäki-Vire *et al.*, 2007). In concordance with this idea, redundant function of FGFR1 and FGFR2 has also been shown in the zebrafish (Leerberg *et al.*, 2019). The activation of canonical FGFs triggers four major signalling pathways: RAS-MAPK, PI3-AKT, PLCγ/protein kinase C, and STAT pathways (Ornitz and Itoh, 2015), and there is evidence suggesting FGF also signals via nuclear FGFR localization (Förthmann *et al.*, 2015).

FGF-signalling in the MHB region induces various genes that modulate the pathway, many of which that are members of a *Fgf8* syn-expression group that is well conserved in vertebrates (Eblaghie *et al.*, 2003; Fürthauer *et al.*, 2002; Haines *et al.*, 2006; Hirate and Okamoto, 2006; Li *et al.*, 2007; Lin *et al.*, 2002; Lin *et al.*, 2005; Minowada *et al.*, 1999; Tsang *et al.*, 2002; Tsang *et al.*, 2004). Positive regulators include the Canopy FGF signalling regulator 1 (*Cnpy1*) and the transmembrane protein FLRT3, which enhances FGF signalling by promoting FGFR maturation in the endoplasmic reticulum (Hirate and Okamoto, 2006), and by direct interaction with the FGFR1 receptor (Böttcher *et al.*, 2004; Haines *et al.*, 2006), respectively. Negative regulators include members of the Sprouty (SPRY) (Yu *et al.*, 2011) and the Dual Specificity Phosphatase (DUSP) (Li *et al.*, 2007) family of proteins, as well as the transmembrane protein Interleukin 17 receptor D (IL17RD) (Lin *et al.*, 2005). SPRY1 and SPRY2 inhibits the RAS-MAPK pathway and regulates PI3K-AKT signalling (Ornitz and Itoh, 2015) while DUSP6 attenuates FGF signalling by dephosphorylation of MAPK (Camps *et al.*, 1998). L17RD blocks the nuclear translocation of activated MAPK and may also inhibit FGF signalling directly interacting with FGFR1 (Fürthauer *et al.*, 2002; Torii *et al.*, 2004; Tsang *et al.*, 2002).

The final transcriptional output of FGF-signalling is mediated by nuclear effectors including the ETV4 and ETV5 transcription factors of the ETS transcription factor family, which regulate the expression of target genes such as *Dusp6* (Ekerot *et al.*, 2008; Znosko *et al.*, 2010). Activation of both positive and negative regulators of FGF signalling is not yet fully understood but may be critical for regulating the timing of active FGF signalling, fine tuning expression patterns, and shaping the FGF signalling gradient in developing tissues, including the MHB.

The role of FGF8 in the IsO

FGF8 is a canonical FGF, and the founding member of a subfamily consisting of FGF8, FGF17, and FGF18 in mice and humans. The zebrafish has 6 members of this family, as the genome contain two paralogous genes for FGF8 (*Fgf8a* and *Fgf8b*) and FGF18 (*Fgf18a* and *Fgf18b*), one gene encoding FGF17, and the additional *Fgf24* gene that has been lost in the tetrapod lineage. The *Fgf8* gene subfamily members have highly dynamic patterns of gene expression during vertebrate development but several of their expression domains are overlapping, including in the MHB (Maruoka *et al.*, 1998; Ohuchi *et al.*, 2000). Still, several lines of evidence suggest that *Fgf8* is the key inducer of IsO activity.

Initial experiments in chick demonstrated that insertion of FGF8-soaked beads in the midbrain exerts similar polarising activity as the IsO, and induces ectopic expression of the MHB markers *En2*, *Pax2*, and *Wnt1* in the midbrain (Crossley *et al.*, 1996; Irving and Mason, 2000; Martinez *et al.*, 1999). In mice, *Fgf8* gene expression in the MHB begins at 4-5 somites, i. e. at an earlier stage than that of *Fgf17* and *Fgf18*, and it is required for the latter´s expression in the region (Chi *et al.*, 2003; Liu *et al.*, 2003). In zebrafish, *Fgf8a* is expressed in the anterior hindbrain at the late gastrula stage, and this expression gradually splits into three anterior rhombomeres, r1, r2, and r4 at early somitogenesis (Reifers *et al.*, 1998). Similar to the situation in mice, zebrafish *Fgf8a* is the most strongly expressed paralogue in the early MHB, while *Fgf8b*, *Fgf18b* and *Fgf24* expression begins slightly later (Jovelin *et al.*, 2010). In mice, removal of *Fgf8* gene expression in the IsO leads to the downregulation of genes in the MHB gene regulatory network, with subsequent progressive aplasia of the posterior midbrain and anterior hindbrain structures (Chi *et al.*, 2003; Meyers *et al.*, 1998), while *Fgf17* and *Fgf18* mutants show mild and no phenotypes, respectively (Liu *et al.*, 2002; Xu *et al.*, 2000). Although the severe phenotype of *Fgf8* MHB knockout mice is caused by increased cell death, moderately reduced levels of FGF-signalling result in changes in cell fate specification (Basson *et al.*, 2008), and zebrafish *Fgf8a* mutants lack cerebellar tissue not because of cell death but transformation of the cerebellar region into midbrain cells (Jászai *et al.*, 2003; Picker *et al.*, 1999; Reifers *et al.*, 1998; Tallafuß and Bally-Cuif, 2003). Thus, *Fgf8* not only affects cell survival but also functions to specify regional identity. The differences in apoptosis between species may be because of species-specific redundancy of other survival factors, e.g., zebrafish WNTs (Buckles *et al.*, 2004). In addition to anteroposterior patterning, *Fgf8*-mediated signalling has been suggested to contribute to the dorsoventral patterning of the MHB in zebrafish and medaka (Carl and Wittbrodt, 1999; Fürthauer *et al.*, 1997), and in mice dorsal MHB structures are more sensitive to reduction of *Fgf8* expression (Chi *et al.*, 2003; Meyers *et al.*, 1998). Thus, these studies have demonstrated the importance of FGF8 for IsO activity and for the polarized gene expression patterns that underlie regionalisation and cell fate choices in the MHB.

Adding to the complexity of FGF8-signalling in the MHB region, multiple isoforms have been identified. In chicken, *Xenopus* and zebrafish, two *Fgf8* isoforms have been described, while eight have been reported in mice, and four in humans (Sunmonu *et al.*, 2011) (Fig. 3A). The two most conserved isoforms, *Fgf8A* and *Fgf8B*, which are present from fish to mammals, exert differential activity in the MHB. While FGF8A regulates midbrain formation (Lee *et al.*, 1997; Liu *et al.*, 1999; Sato *et al.*, 2001), FGF8B also promotes development of the cerebellum (Liu *et al.*, 1999; Sato *et al.*, 2001). A potential explanation for this discrepancy is the higher receptor binding affinity of FGF8B isoform due to the presence of phenylalanine 32 in the N-terminal region (Olsen *et al.*, 2006). Indeed, in both chicken and zebrafish, FGF8A and FGF8B activate FGF signalling with different intensity, and reduced concentrations of *Fgf8b* can mimic the effect of *Fgf8a* (Inoue *et al.*, 2006; Sato *et al.*, 2001). However, even at very high concentrations, electroporation of *Fgf8a* cannot mimic FGF8B activity and induce cerebellar development (Fletcher *et al.*, 2006; Sunmonu *et al.*, 2011), and in both mouse and chicken only FGF8B can ectopically induce hindbrain genes such as *Gbx2* in the midbrain region (Liu *et al.*, 1999; Sato *et al.*, 2001). In addition, it was shown that only FGF8B misexpression activates the Ras–Erk pathway in the midbrain of the chick (Sato and Nakamura, 2004). These results suggest that differences in signalling properties are both quantitative and qualitative, and this interpretation is further supported by data from mouse genetic experiments. Removal of all *Fgf8B*-containing isoforms in mice phenocopies the complete deletion of *Fgf8*, leading to downregulation of key IsO regulatory genes, and subsequent loss of the MHB-derived structures (Guo

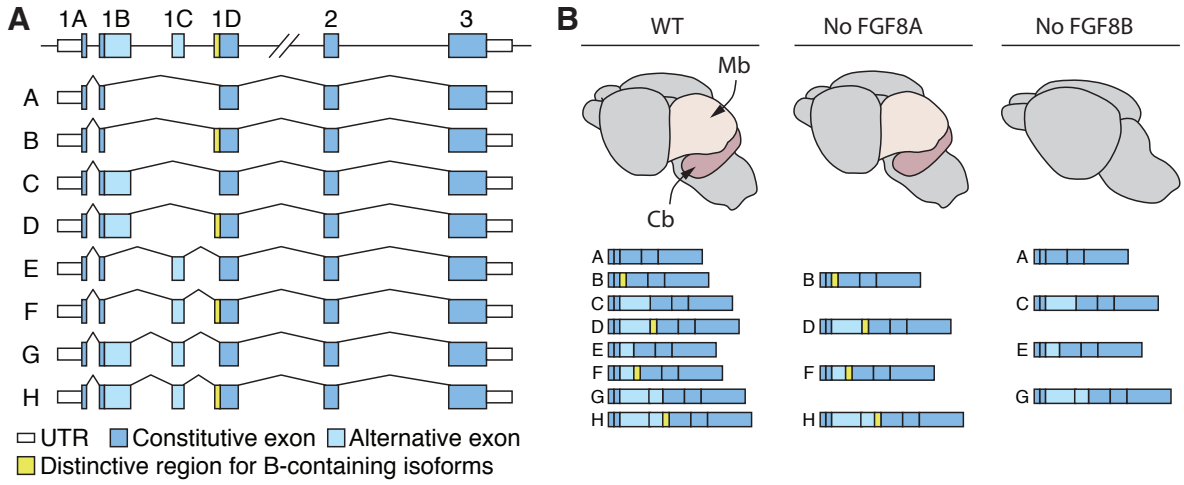

Fig. 3. Differential requirement for *Fgf8* isoforms in the midbrain-hindbrain boundary (MHB). (A) Representation of all Fgf8 isoforms reported in the mouse. Isoforms C, D, G, and H are not present in humans due to a premature stop codon in exon 1B. Exon 1C is only present in placental mammals, while *Fgf8A* and *Fgf8B* are evolutionarily conserved across vertebrates, from fish to mammals. **(B)** Schematic illustration of e18.5 brains from wildtype controls, mutant mice with a splice-acceptor mutation that removes all *Fgf8A*-containing isoforms (middle), or with a splice-acceptor mutation that remove all Fgf8B-containing isoforms (right). Only the removal of *Fgf8B*-containing isoforms results in the loss of posterior midbrain structures and cerebellar aplasia, mimicking the phenotype observed in MHB *Fgf8* KO mice. Cb: cerebellum; Mb: midbrain.

et al., 2010) (Fig. 3B). In contrast, even though removal of *Fgf8A* isoforms leads to growth retardation and perinatal lethality, MHB patterning and growth are not affected, including when overall FGF-signalling is reduced by the additional removal of *Fgf17* expression (Guo *et al.*, 2010) (Fig. 3B). Thus, these data demonstrated that diverse *Fgf8* isoforms exert distinct signalling strength, but also highlighted likely differences in the downstream signal transduction cascade and established that *Fgf8b* is the main isoform mediating IsO activity *in vivo*. Despite them having different signalling properties and apparently distinct roles in specific tissues, there is little, if any, differential regulation of *Fgf8* isoforms during development either temporal or spatially: multiple isoforms are expressed simultaneously in different tissues (Blunt *et al.*, 1997). Still, the spatio-temporal regulation of *Fgf8* gene expression is tightly controlled in the vertebrate embryo, including in the MHB.

The regulatory landscape of Fgf8

In vertebrates, the precise expression of many developmental genes, including *Fgf8*, is regulated by multiple cis-regulatory elements. Beyond the promoter regions near the target genes, distant transcriptional enhancers play a crucial role in activating the specific temporal and spatial expression patterns of these genes (Long *et al.*, 2016). Although these enhancers can be located within or beyond neighbouring genes and influence gene expression across large genomic distances (Lettice *et al.*, 2003), physical proximity between enhancers and their target promoters is crucial for gene activation (Chen *et al.*, 2024; Zuin *et al.*, 2022). In most animals, the genome is partitioned into so-called topologically associated domains (TADs) (Acemel and Lupiáñez, 2023), which consist of segmental chromosomal regions that are primarily self-interacting (Dixon *et al.*, 2012; Nora *et al.*, 2012; Sexton *et al.*, 2012). TADs largely overlap with gene regulatory domains (Symmons *et al.*, 2014) and facilitate enhancer-promoter interactions by increasing the probability of physical contact.

Concurrently, TADs limit the genomic range in which enhancers can act and thus reduce the probability of ectopic activation of non-target genes. This restriction imposes evolutionary constraints, as disrupting this organization can result in gene misexpression and cause severe developmental malformations or disease (Flavahan *et al.*, 2016; Franke *et al.*, 2016; Lupiáñez *et al.*, 2015; Rajderkar *et al.*, 2023; Symmons *et al.*, 2016). Consequently, TADs are more often reorganized as intact modules during genome evolution (Vietri Rudan *et al.*, 2015; Farré *et al.*, 2015; Li *et al.*, 2022).

Similarly, since regulatory sequences can be located in neighbouring genes or even further away, cis-regulatory constraints contribute to shaping conservation of syntenic regions (Irimia *et al.*, 2012; Kikuta *et al.*, 2007). Ancient conservation of microsynteny has been used to identify genomic regulatory blocks (GRBs) that are regions of the genome containing developmental genes that are physically linked to nearby bystander genes because they are part of their gene regulatory landscape (Irimia *et al.*, 2012; Kikuta *et al.*, 2007). The boundaries of GRBs are predictive for TAD boundaries and GRBs tend to overlap with TADs (Harmston *et al.*, 2017).

In mice and humans, *Fgf8* is located in a syntenic region that also contains *Tlx1*, *Lbx1*, *Btrc*, *Poll*, *Dpcd, Fbxw4*, and *Npm3* (Fig. 4A). In zebrafish *Btrc*, *Poll*, *Dpcd and Npm3* genes have been lost from the locus, while the *Fgf8a*/*Fbxw4* gene pair is inverted and the *Slc2a15a* gene intercalates between *Lbx1* and *Fbxw4* (Fig. 4A). The microsynteny of the *Fgf8*/*Fbxw4* gene pair is ancient and conserved in both the hemichordate *S. kowalevskii* (Simakov *et al.*, 2015) and the urochordate *C. intestinalis* (Jovelin *et al.*, 2010) suggesting that they form an ancient GRB (Cañestro *et al.*, 2007) (Fig. 4A). Corroborating this regulatory linkage, in mice *Fbxw4* and *Fgf8* are the only genes that are located within the same TAD, and this region also largely overlaps the *Fgf8* regulatory domain (Cova *et al.*, 2023; Marinić *et al.*, 2013). Given the fundamental importance of *Fgf8* in chordate development, one could speculate that distant regulatory elements directing *Fgf8* gene expression were present within the *Fbxw4* gene already in very early chordates, and that loss

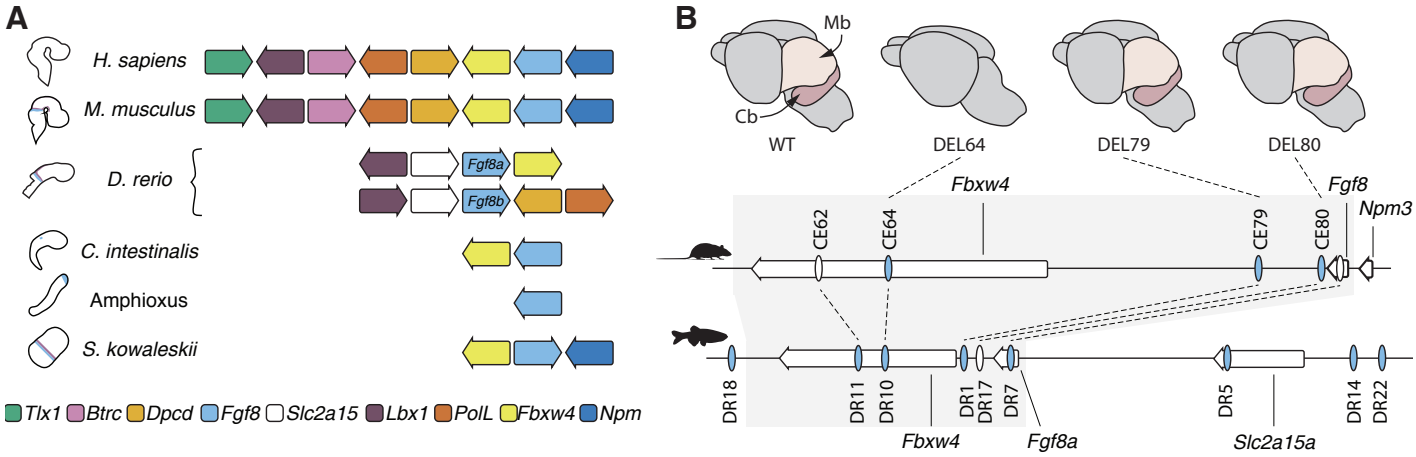

Fig. 4. The regulatory landscape of *Fgf8* is embedded within an ancient gene regulatory block. (A) Representation of the synteny of the *Fgf8*-containing genomic region in deuterostome species. The *Fbxw4-Fgf8* gene pair is retained in all species with an IsO-like expression of *Fgf8*. **(B)** Putative midbrain-hindbrain boundary (MHB) enhancers (ovals) in the *Fgf8* genomic region in mouse and zebrafish. Blue indicates reported MHB expression in transgenic assays. Grey box depicts the *Fbxw4-Fgf8* syntenic region that corresponds to the mouse topologically associated domains (TAD) and that is conserved in the deuterostome lineage. The upper row illustrates e18.5 mouse brains: wildtype (left), CE64 enhancer deletion mutants (middle left), CE79 mutants (middle right), and CE80 mutants (right). The removal of CE64 results in complete absence of *Fgf8* expression in the MHB, and subsequent loss of the posterior midbrain and cerebellum. Cb: cerebellum; Mb: midbrain.

of the *Fgf8*/*Fbxw4* synteny in amphioxus is related to the complete lack of an IsO in this species (Fig. 4A).

The enhancer landscape of the *Fgf8* locus has been extensively studied in both mice and zebrafish, and several putative enhancers with tissue-specific regulatory activity have been identified (Beermann *et al.*, 2006; Hörnblad *et al.*, 2021; Hu *et al.*, 2004; Inoue *et al.*, 2006; Komisarczuk *et al.*, 2009; Marinić *et al.*, 2013; Sasaki *et al.*, 2008). Most of these putative enhancers are located downstream of the *Fgf8* gene, in the intergenic region between *Fbxw4* and *Fgf8,* or embedded within the *Fbxw4* gene. These enhancers frequently have overlapping tissue-specific regulatory activity so that multiple elements can drive expression in similar spatio-temporal domains (Marinić *et al.*, 2013). Such redundancy confers robustness to gene expression and allows for evolution of regulatory novelty (Frankel *et al.*, 2010; Hong *et al.*, 2008; Hörnblad *et al.*, 2021; Osterwalder *et al.*, 2018; Perry *et al.*, 2010)

A large reporter screen of human conserved elements identified three putative MHB enhancers (CE80, CE79, and CE64) that could drive reporter expression in the MHB region of mice at e10.5 (Marinić *et al.*, 2013). Two of these elements, CE80 and CE79, are located in a region proximal to the 3' end of the *Fgf8* gene and had been identified previously in a similar screen (Beermann *et al.*, 2006) (Fig. 4B). The distal CE64 is located 120kb downstream of *Fgf8* in the 4th intron of *Fbxw4* (Fig. 4B). All of these putative enhancers are highly conserved from fish to humans, and were also identified as putative enhancers in the zebrafish (Inoue *et al.*, 2006; Komisarczuk *et al.*, 2009). However, in zebrafish, only CE79 and CE64 showed regulatory activity in the MHB in reporter assays, while CE80 was active in other tissues (Fig. 4B). In addition, five other putative MHB enhancers were also reported in zebrafish (Komisarczuk *et al.*, 2009) (Fig. 4B). Importantly, *in vivo* enhancer deletions demonstrated that in the mouse only the most distal CE64 MHB enhancer is essential for *Fgf8* expression; its removal results in complete failure to induce Fgf8 expression in the MHB, with subsequent cerebellar aplasia and loss of posterior midbrain structures (Hörnblad *et al.*, 2021) (Fig. 4B). In contrast, single deletions of the more proximal CE80 and CE79, or deletion of both together caused only a very small reduction in Fgf8 expression when CE80 was absent, and animals lacking C79 or CE80 (or both) developed normally (Hörnblad *et al.*, 2021) (Fig. 4B). Although these experiments do not exclude the possibility that the CE80 and CE79 enhancers may function redundantly with CE64 in the MHB maintenance phase, they established CE64 as an essential main enhancer required for both initiation and maintenance of *Fgf8* expression in the IsO. In zebrafish, functional data on the *in vivo* importance of putative enhancers are incomplete, but detailed transgenic reporter experiments have shown that the zebrafish equivalent to CE79 cannot recapitulate the very early expression of *Fgf8a* in the MHB (Inoue *et al.*, 2008). This observation suggests that, similar to the mouse, CE79 has a less important role for initiation of *Fgf8a* expression. Interestingly, the synteny of *Fgf8/Fbxw4* has been lost in the *Fgf8b* locus, and this paralogue only becomes expressed after *Fgf8a* in the MHB. This may indicate that enhancers within the *Fbxw4* gene are important for initiation of *Fgf8a* expression, also in zebrafish. Taken together, these data highlight the need for functional *in vivo* characterization of putative regulatory elements in several species to fully understand the complexity of the *Fgf8* regulatory logic in the MHB and how it has evolved.

Many conserved genetic interactions in the core MHB gene regulatory network have been described, mainly in the positioning and maintenance of the IsO. Less is known about the induction of the MHB, and the direct regulatory interactions between transcription factors and regulatory elements at specific loci that initiate and maintain the MHB gene expression programme. Although it was reported that PAX2 can bind to CE79 in zebrafish (Inoue *et al.*, 2008), and phylogenetic footprinting together with *in vivo* functional dissection of the mouse CE64 enhancer could identify essential regulatory motifs (Hörnblad *et al.*, 2021), no additional data have demonstrated the direct interaction between trans-acting transcription factors and their cognate *Fgf8* enhancers in the MHB.

Discussion

Fgf8 signalling emanating from the IsO coordinates the patterning, differentiation, and growth of the vertebrate MHB region. This makes the IsO a prime model to explore how localised gene expression drives large-scale developmental processes, thereby ensuring stereotypic patterning and morphogenesis during embryonic development. The ancient origin of the gene regulatory networks that direct the formation and function of the MHB-region also makes it a valuable model for studying evolutionary conservation and plasticity in gene regulation.

A wealth of studies has identified a network of genes involved in regulating IsO formation, maintenance and function, and the morphogenetic processes that accompany these gene expression programmes have also been described. Less is known about the cell-type specific, genome-wide, and direct regulatory interactions, which occur in the context of the topological organisation of the genome. Thus, the direct transcriptional regulators of *Fgf8* in the MHB are not known, and a full understanding of the cis-regulatory sequences through which they act is still missing. A critical step will therefore be to identify these transcription factors and to understand their specific roles in initiating and maintaining *Fgf8* MHB gene expression. Identification of the transcription factors will help in the decoding of the complex regulatory logic of *Fgf8* and reveal how the GRN have been evolutionarily conserved and adapted across vertebrates.

While key enhancers have been functionally validated in mice, more comprehensive *in vivo* functional characterization across diverse species is required to unravel the evolutionary dynamics of *Fgf8* regulation. Despite the sequence conservation of putative *cis*-regulatory elements from zebrafish to human, the temporal activity of these enhancers and their interactions with trans-acting factors may differ across species. Furthermore, it remains to be understood whether the deep conservation of these putative enhancers reflects a rigid 'enhanceosome'-like regulatory architecture or whether their organisation is more flexible and allows for reshuffling of regulatory motifs according to the ´billboard' model (Kulkarni and Arnosti, 2003; Thanos and Maniatis, 1995). In mice, the presence of one essential MHB enhancer containing both redundant and non-redundant regulatory features suggests a mixture of the two (Hörnblad *et al.*, 2021). Similar *cis*-regulatory architecture could potentially hold true for other species such as zebrafish, but the high number of putative enhancers driving reporter expression in the MHB in this species may also indicate a distinct, and more distributed regulatory architecture. Still, it is clear that the regulatory potential of a developmental enhancer, as demonstrated by transgenic reporter assays, does not necessarily reflect its relative importance in the tissue *in vivo*.

The implementation of CRISPR-based enhancer deletions and the generation of highly precise transgenic reporter lines will help in the exploration of the spatiotemporal control of key MHB genes, including *Fgf8*. Integrating these genetic tools with innovative techniques, such as chromatin conformation capture and single-cell sequencing technology, will help to unravel the wiring of the MHB gene regulatory network, and in particular the interactions between trans-acting factors and their target cis-regulatory elements. By exploring the genome-wide regulatory landscapes and gene expression profiles of distinct cell types at the MHB, as well as identifying the transcription factor-enhancer interactions that direct midbrain and hindbrain development, we can gain significant insights into the molecular orchestration of vertebrate brain patterning, development and evolution.

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