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Developing roles for Hox proteins in hindbrain gene regulatory networks

PRIYANJALI GHOSH and CHARLES G. SAGERSTRÖM*

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Massachusetts, USA

ABSTRACT Hox proteins have long been known to function as transcriptional regulators during development of the vertebrate hindbrain. In particular, these factors are thought to play key roles in assigning distinct fates to the rhombomere segments arising in the embryonic hindbrain. However, it remains uncertain exactly how the Hox proteins fit into the regulatory networks controlling hindbrain formation. For instance, it is unclear if Hox proteins fulfill similar roles in different rhombomeres and if they are absolutely required for all aspects of each rhombomere fate. Recent advances in the discovery, characterization and functional analysis of hindbrain gene regulatory networks is now allowing us to revisit these types of questions. In this review we focus on recent data on the formation of caudal rhombomeres in vertebrates, with a specific focus on zebrafish, to derive an up-to-date view of the role for Hox proteins in the regulation of hindbrain development.

KEY WORDS: *rhombomere, transcription, segmentation, embryogenesis*

Hox factors act during rhombomere formation in the vertebrate hindbrain

A role for *Hox* genes in regulating establishment of the anteroposterior body axis was first uncovered in the fruit fly and an equivalent role was subsequently confirmed in vertebrates (reviewed in (Mallo *et al*., 2010). The most dramatic manifestation of *Hox* function in vertebrate axis formation can be observed in the embryonic hindbrain (Krumlauf, 2016). In particular, the early vertebrate hindbrain primordium is transiently divided into seven or eight segments (rhombomeres; Fig. 1A) and *Hox* genes are required for establishing at least rhombomere 2 (r2) through r7/r8. Each rhombomere represents a unique set of cell fates and is the source of distinctive neural progenitors that ultimately differentiate into cell segment-specific neurons (Fig. 1A). In particular, several rhombomeres contribute neurons to the cranial nerves, such that trigeminal neurons form in r2 and r3, facial neurons in r4, abducens neurons in r5 and r6, and vagal neurons in the caudal-most portion of the hindbrain. Additionally, reticulospinal neurons arise in a rhombomere-specific pattern with Mauthner neurons, which are particularly prominent in aquatic species, forming in r4. Via these classes of neurons, the hindbrain is responsible for regulating complex physiological processes such as breathing, heartbeat, circulation and wakefulness, as well as to ensure innervation of the face, head, and neck. Furthermore, the cerebellum arises from the dorsalmost region of r1 and plays an essential role in motor control, as well as some cognitive functions. Hence, proper hindbrain segmentation is essential for correct fate specification and appropriate anatomical positioning of key types of neurons and neural structures. Disruptions to these processes leads to incomplete neural circuits and abnormal neural function. For instance, mutations in human *HOXA1* leads to structural defects of the brainstem that have been associated with autism spectrum disorders (Ingram *et al*., 2000; Tischfield *et al*., 2005).

Segmentation of the hindbrain primordium starts shortly after gastrulation is completed. In zebrafish, this process begins with the formation of r4, followed by formation of r1/r2, r3, r7, and r5/r6 (Moens *et al*., 1998), but the order of rhombomere establishment varies between species (Lumsden, 1990). *Hox* genes play a key role in this process – in particular, *Hox* genes of paralog groups 1-4 (PG1-4) are active in the developing hindbrain. In spite of having undergone an additional genome duplication (Fig. 1 B,C), the zebrafish genome does not contain duplicate copies of *Hox* genes in PG1-4, except for having two *Hoxb1* genes (Amores *et al*., 1998). In this case, zebrafish *hoxb1a* remains functionally analogous to murine *Hoxb1*, while zebrafish *hoxb1b* has taken the role of murine *Hoxa1* (McClintock *et al*., 2001). The first *Hox* genes (*Hoxa1* in the mouse and *hoxb1b* in zebrafish) are expressed during gastrulation and are transcribed in a caudal domain with their anterior limit at

Abbreviations used in this paper: Fgf, fibroblast growth factor; r, rhombomere; RA, retinoic acid; TF, transcription factor.

***Address correspondence to:** Charles Sagerström. Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St/LRB815, Worcester, MA 01605, USA. Tel: +1(508)-856-8006. E-mail: charles.sagerstrom@umassmed.edu - https://orcid.org/0000-0002-1509-5810
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the future r3/r4 boundary (Alexandre *et al.*, 1996; Murphy and Hill, 1991; Sagerström *et al.*, 2001). Shortly thereafter, *Hoxb1* (*hoxb1a* in zebrafish) becomes expressed in a domain that coincides with the future r4 (Murphy *et al.*, 1989; Prince *et al.*, 1998; Wilkinson *et al.*, 1989). Accordingly, the paralog group 1 *Hox* genes (*Hoxa1/hoxb1b* and *Hoxb1/hoxb1a*) are required for formation of r4, but they play distinct roles. In particular, *Hoxa1/hoxb1b* mutants have a smaller r4 (as well as a larger r3), while *Hoxb1/hoxb1a* mutants have a mis-specified r4 (where facial motor neurons fail to migrate out of r4 (Carpenter *et al.*, 1993; Chisaka *et al.*, 1992; Gavalas *et al.*, 1998; Goddard *et al.*, 1996; Lufkin *et al.*, 1991; Mark *et al.*, 1993; Rossel and Capecchi, 1999; Selland *et al.*, 2018; Studer *et al.*, 1996; Weicksel *et al.*, 2014) and *hoxb1a* mutant zebrafish also lack the r4-specific Mauthner neurons (Selland *et al.*, 2018; Weicksel *et al.*, 2014). *Hoxb2* (*hoxb2a* in zebrafish) is expressed in r3-r5 and mutations in this gene disrupt r4 specification such that formation of the facial motor neurons is disrupted (Barrow and Capecchi, 1996), but does not appear to affect the formation of other rhombomeres. In contrast, mutations in *Hoxa2* (*hoxa2b* in zebrafish), which is expressed in r2-r5, does not affect segmentation or specification of the corresponding rhombomeres (Hunter and Prince, 2002; Rijli *et al.*, 1993). Additionally, mutations in paralog group 3 *Hox* genes (particularly simultaneous loss of *Hoxa3* and *Hoxb3*) disrupt formation of the abducens motor nucleus in murine r5 (Gaufo *et al.*, 2003). Hence, *Hox* genes play key roles in segmentation (*Hoxa1/hoxb1b*) and specification (*Hoxb1/hoxb1a, Hoxb2/hoxb2a, Hoxa3* and *Hoxb3*) of several rhombomeres. Several non-Hox transcription factors (TFs) are also involved in hindbrain specification. For instance, the *MafB* (a.k.a. *kreisler* in mouse and *valentino* in zebrafish) and *Hnf1b* (*hnf1ba* in zebrafish) TFs are required for formation of r5/r6 (Cordes and Barsh, 1994; Frohman *et al.*, 1993; F. A. Kim *et al.*, 2005; Moens *et al.*, 1998; Sun and Hopkins, 2001), while the *Egr2/Krox20* TF is necessary for establishment of r3 and r5 (Schneider-Maunoury *et al.*, 1993). Similarly, *iroquois* family TFs are required for the establishment of more anterior rhombomeres (Itoh *et al.*, 2002; Stedman *et al.*, 2009). In addition, at least two morphogens are essential to drive expression of TFs during hindbrain development. Retinoic acid (RA) is produced by dorsal mesoderm during gastrula stages and is responsible for the activation of several hindbrain genes (including the *Hox* genes). RA is thought to exist in a gradient, with higher concentrations posteriorly, and to turn on the expression of different TFs in distinct rhombomeres in response to this concentration gradient (Shimozono *et al.*, 2013). Fibroblast growth factors (Fgfs) 3 and 8 are also produced in the hindbrain, specifically at the midbrain-hindbrain boundary and in r4 (Maves *et al.*, 2002; Walshe *et al.*, 2002). Several of these factors also function together such that, for instance, Fgf cooperates with Hnf1ba to

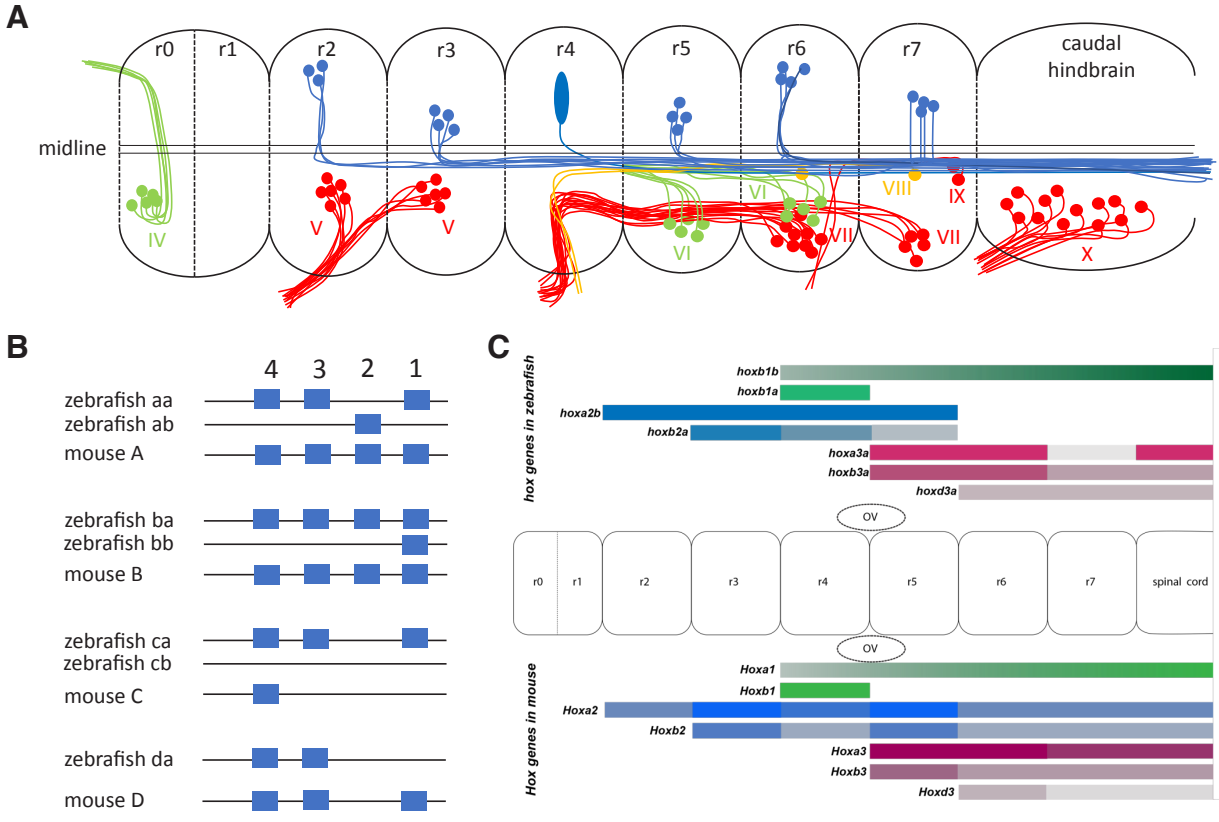

Fig. 1. *Hox* genes control vertebrate hindbrain formation. (A) *Segmental arrangement of neurons in the zebrafish hindbrain. Reticulospinal neurons (blue) and their projections are shown above the midline and neurons contributing to the cranial nerves are shown below the midline. Green, somatic motor neurons (cranial nerves IV and VI); red, branchiomotor neurons (cranial nerves V, VII, IX and X); yellow, otic efferent neurons (cranial nerve VIII). Diagram is drawn with anterior to the left. r, rhombomere.* **(B)** *Genomic organization of paralog group 1-4* Hox *genes in zebrafish and mouse. Note that the zebrafish genome has undergone an additional duplication relative to the mouse genome. Most of these duplicate genes have been lost, such that only* hoxb1 *is found to have two copies in present day zebrafish.* **(C)** *Extent of* Hox *gene in the zebrafish (top) and mouse (bottom) hindbrain.*

activate downstream genes in r5/r6 (Hernandez *et al.*, 2004; Wiellette and Sive, 2003).

Hox proteins act with TALE factors to control hindbrain gene expression

After *Hox* genes were initially cloned, it became clear that they encode proteins containing a helix-turn-helix DNA binding motif – the homeobox (reviewed in (Gehring *et al.*, 1994) – suggesting that they function as transcription factors to control gene expression (reviewed in (Levine and Hoey, 1988). However, subsequent detailed biochemical analyses revealed that Hox proteins have poor affinity and specificity for DNA, with most Hox proteins preferring to bind AT-rich sequences. Accordingly, Hox proteins function in complexes with other TFs that facilitate their binding to DNA and that ensure greater sequence selectivity (reviewed in (Ladam and Sagerström, 2014; Mann *et al.*, 2009). In particular, Hox proteins bind genomic DNA in complexes with members of the TALE (three amino acid loop extension) family of homeodomain proteins. Combining data from mouse and zebrafish, the TALE family includes at least four Pbx, four Meis and three Prep TFs that can interact with Hox proteins – where the Prep and Meis proteins are more closely related to each other than to Pbx (Bürglin and Affolter, 2016). Many Hox proteins bind Pbx TFs using a short motif (YPWM) found N-terminal to the Hox homeodomain (reviewed in (Mann and Chan, 1996), while other Hox proteins (particularly the Abd-B paralogs (Shen *et al.*, 1997; Williams *et al.*, 2005) bind members of the Meis/Prep family via N-terminal sequences in the Hox protein. Notably, Pbx and Prep/Meis form heterodimers, meaning that TALE TFs can be part of a Hox transcription complex not only by binding Hox proteins directly, but also via their interactions with each other. As a result, many Hox-controlled regulatory elements have been shown to be occupied by trimeric Prep (or Meis):Pbx:Hox complexes (Berthelsen *et al.*, 1998; Ferretti *et al.*, 2000; Jacobs *et al.*, 1999; Ryoo *et al.*, 1999; Shanmugam *et al.*, 1999; Shen *et al.*, 1999; Vlachakis *et al.*, 2001; 2000). In particular, trimeric complexes containing the earliest expressed Hox proteins in the hindbrain (HoxA1/Hoxb1b) are required for the initial expression of hindbrain-specific genes such as *Hoxb1*/*hoxb1a* and *Hoxb2/hoxb2a*.

Our understanding of Hox function during r4 and r5/r6 formation is incomplete

Based on the studies discussed above, regulatory pathways controlling formation of the hindbrain have been derived. In particular, formation of r4-r6 in the caudal hindbrain has served as an informative model for rhombomere formation in general (Fig. 2). In this model, hindbrain-specific gene expression is initiated by RA signaling during gastrulation. In particular, RA binds and activates a heterodimeric complex of RA receptors (RARs) and retinoic X receptors (RXRs) that enters the nucleus and targets genomic regulatory sequences known as RA response elements (RAREs; reviewed in (Mark *et al.*, 2006). RAREs are present in the *Hox* clusters (Soshnikova, 2014), where RA promotes decondensation of otherwise compacted chromosomal chromatin, thereby permitting active transcription (Chambeyron and Bickmore, 2004; Chambeyron *et al.*, 2005). RA is also required for expression of *hnf1ba* and *MafB/valentino* – the earliest-expressed TFs in r5/r6 (Dupé and Lumsden, 2001; Hernandez *et al.*, 2004; Wendling *et al.*, 2001), further demonstrating the central role of this morphogen in the initiation of hindbrain gene expression. Once hindbrain gene expression has been initiated by RA, it is thought that the earliest TFs sit at the top of regulatory pathways to direct rhombomere-specific gene expression. In particular, by combining data from mouse and zebrafish, a *Hox*-regulated pathway is postulated to be operative in r4 such that HoxA1/Hoxb1b first acts (together with TALE factors) to initiate *Hoxb1/hoxb1a* expression (Vlachakis *et al.*, 2001). *Hoxb1/hoxb1a* expression is then maintained by an autoregulatory loop in r4 where HoxB1/Hoxb1a (also acting with TALE factors) binds its own regulatory elements to sustain its expression (Ferretti *et al.*, 2000; 2005; Pöpperl *et al.*, 1995). Both HoxA1/Hoxb1b and HoxB1/Hoxb1a can also bind regulatory elements at the *Hoxb2/hoxb2a* locus to drive expression of this gene in r4 (Ferretti *et al.*, 2000; Jacobs *et al.*, 1999). In an analogous fashion, *Hnf1b/hnf1ba* is induced by RA in r5/r6 (Hernandez *et al.*, 2004), where it subsequently acts with Fgf to induce *MafB/valentino* expression (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). MafB/Valentino in turn activates *Egr2/Krox20* expression in r5 (Frohman *et al.*, 1993; Moens *et al.*, 1998). In r5/r6, the paralog group 3 (PG3) *Hox* genes (specifically *Hoxa3/hoxa3a, Hoxb3/hoxb3a* and *Hoxd3/hoxd3a*) are also predicted to function downstream of *MafB/valentino* (Frohman *et al.*, 1993; Moens *et al.*, 1998; Prince *et al.*, 1998). Hence, relatively linear and hierarchical gene regulatory pathways have been postulated for both r4 and r5/r6 specification. However, several observations suggest that the situation is likely to be more complex. Specifically, *hoxb1a* is still expressed in the residual r4 domain of *hoxb1b* mutant zebrafish (Selland *et al.*, 2018; Weicksel *et al.*, 2014), suggesting that *hoxb1a* expression is not completely dependent on *hoxb1b*. Some *hoxb1b* mutant zebrafish also retain the r4-specific Mauthner neurons (Weicksel *et al.*, 2014) and *hoxb2a* depletion does not affect r4 formation (Hunter and Prince, 2002), suggesting that none of the *Hox* genes expressed in r4 may be absolutely required for r4 specification. Furthermore, recent ChIP-seq analyses in zebrafish embryos identified Hoxb1b binding near several genes expressed in r5/r6 (e.g. *celf2, nr2f2* and *mmp2*; Stanney *et al.*, in preparation), suggesting that r4 and r5/r6 specification may be regulated by

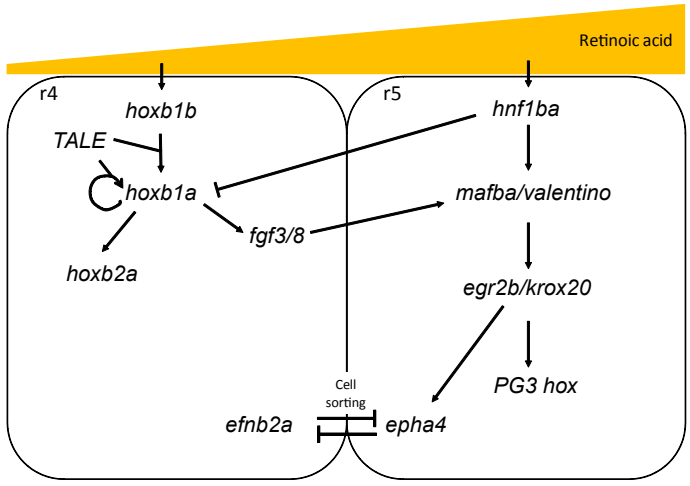

Fig. 2. A previous view of regulatory pathways controlling r4 (left) and r5 (right) formation. *The diagram is based on zebrafish data and relevant comparisons to the mouse are made in the text.*

the same factors – at least at the earliest stages of development. Indeed, since *Hoxa1/hoxb1b* is transiently expressed throughout the caudal hindbrain, it is possible that this TF is required for both r4 and r5/r6 formation. Hence, it appears likely that the regulatory pathways for r4-r6 formation are more complex than initially thought.

Derivation of expanded gene regulatory networks for r4 and r5/r6

Given the likely complex nature of the pathways controlling r4 and r5/r6 formation, it is unclear why additional key regulatory factors have not been identified in these pathways. Part of the explanation may stem from the manner in which such key factors were originally identified – either in forward genetic screens or as a result of candidate approaches. In particular, *MafB/valentino* and *Hnf1b/hnf1ba* where identified in genetic screens in mouse and zebrafish (Frohman *et al*., 1993; Moens *et al*., 1996; Sun and Hopkins, 2001), while the *Hox* genes and *Egr2/Krox20* were identified as potential regulators based on their known roles in other systems. Subsequent targeted mutagenesis of these genes confirmed their important functions in r4 and/or r5/r6 formation (Barrow and Capecchi, 1996; Carpenter *et al*., 1993; Chisaka *et al*., 1992; Gaufo *et al*., 2003; Gavalas *et al*., 1998; Goddard *et al*., 1996; Lufkin *et al*., 1991; Mark *et al*., 1993; Rijli *et al*., 1993; Rossel and Capecchi, 1999; Selland *et al*., 2018; Studer *et al*., 1996; Weicksel *et al*., 2013; Zigman *et al*., 2014). However, both of these strategies have shortcomings in terms of identifying the complete set of genes controlling rhombomere formation. Specifically, it has been estimated that the forward genetic screens in zebrafish reached only 25% (insertional screens) to 50% (ENU screens) saturation of the genome (Amsterdam *et al*., 2004; Haffter *et al*., 1996) and the candidate approaches are limited to assessments of genes with known functions in other systems. Hence, it seems likely that important regulators of rhombomere formation remain to be identified.

The advent of less biased high-throughput approaches for gene discovery and functional analyses have recently led to renewed efforts aimed at identifying additional factors controlling rhombomere formation. First, differential gene expression analyses (by microarray, RNA-seq, etc.) have been used to compare the transcriptional profiles of tissues with ectopic or disrupted expression of early-acting TFs – particularly *Hox* TFs (Bami *et al*., 2011; Choe *et al*., 2011; De Kumar *et al*., 2017; Gouti and Gavalas, 2008; Rohrschneider *et al*., 2007; van den Akker *et al*., 2010) – as well as to compare expression profiles between rhombomeres (Chambers *et al*., 2009), and the resulting gene sets have been deposited in community-based gene expression databases. Second, novel genome editing approaches have enabled functional analyses of larger numbers of candidate genes. Specifically, zinc finger nucleases (ZFNs), TALE (Transcription activator-like effector) nucleases (TALENs) and CRISPR/Cas9 approaches permit relatively rapid mutagenesis of individual genes (Jinek *et al*., 2012; Y. G. Kim *et al*., 1996; Mali *et al*., 2013; Miller *et al*., 2011) while the TILLING (Targeted Induced Local Lesions) reverse genetics strategy (McCallum *et al*., 2000; Wienholds *et al*., 2003) has been used to generate mutants for known genes on a larger scale. By making use of these higher throughput tools, it is now possible to identify and functionally characterize novel candidate genes for involvement in r4 and r5/r6 formation. For example, one recent effort made use of the Zebrafish Information Network (ZFIN) gene expression database and identified 107 genes as being restricted to one, or several, rhombomeres in the r4-r6 region of the hindbrain (Ghosh *et al*., 2018). Thirty-nine of these genes are expressed in r4 and 68 in r5/r6, demonstrating that the regulatory networks controlling formation of these rhombomeres are relatively large. Furthermore, ~80% of these genes had not been previously analyzed in the context of hindbrain development, indicating that many of these genes may represent novel regulators.

Hox proteins play different roles in the r4 and r5/r6 gene regulatory networks

While the various strategies discussed above have led to the identification of novel genes expressed in r4 and r5/r6 in several species, it is also necessary to determine how these genes function in a regulatory network to control rhombomere formation. In an attempt to accomplish this, a subset of the 39 genes expressed in r4 was assessed in zebrafish mutants for the PG1 *Hox* genes (Ghosh *et al*., 2018). If the PG1 genes reside at the top of an r4 regulatory cascade, as predicted from the model in Fig. 2, expression of most r4 genes should be lost in PG1 *Hox* mutants. Strikingly, of 14 r4 genes tested in this manner, all remained expressed in both *hoxb1b* and *hoxb1a* zebrafish mutants. Another recent report arrived at a similar conclusion while assaying expression of a smaller set of genes (Selland *et al*., 2018) – although these authors detected subtle changes in gene expression for some of the r4 genes in *hoxb1b* mutants. These findings demonstrate that, contrary to a hierarchical model for r4 gene regulation, PG1 *Hox* genes are not absolutely required for expression of most r4 genes. Similarly, disrupting RA signaling does not block r4 gene expression in the zebrafish embryo, which contradicts the view that RA is required for r4 formation because it activates PG1 *Hox* expression in the hindbrain primordium. Notably however, when RA signaling is blocked in *hoxb1b* mutant zebrafish embryos, expression of all tested r4 genes is lost (Ghosh *et al*., 2018). Fgf signaling is also required for r4 formation, but in a different fashion. In particular, disrupting Fgf signaling blocks expression only of genes that are part of the Fgf signaling pathway itself (Ghosh *et al*., 2018; Selland *et al*., 2018). Furthermore, the integration of novel genes into the r4 network revealed previously unknown repressive interactions. For instance, *hoxb1a* is required to repress *gbx1* expression in r4 (Ghosh *et al.,* 2018). Hence, the r4 regulatory network is not a linear pathway, but most r4 genes are under joint control of *hoxb1b* and RA, while Fgf signaling functions in a subprogram within a larger r4 regulatory network.

Similar to the situation in r4, both RA and Fgf are required for gene expression in r5/r6, but these signals act in a different manner in this region. In particular, RA is necessary and sufficient for expression of *hnf1ba* (Hernandez *et al*., 2004) – the earliest acting TF in r5/r6 – in zebrafish. Furthermore, mutations in *hnf1ba,* as well as in *MafB/valentino* – the TF acting immediately downstream of *hnf1ba* – are sufficient to disrupt the expression of all r5/r6 genes, including ten novel r5/r6 genes identified from the zebrafish gene expression database (Ghosh *et al*., 2018). This effect of *hnf1ba* and *MafB/valentino* mutations is in contrast to r4, where mutations in the PG1 *Hox* genes do not affect gene expression. *hnf1ba* also represses expression of some, but not all, r4 genes thereby aiding in the establishment of the r4/r5 boundary. Hence, RA and the earliest-acting TFs cooperate in r4, but not in r5/r6. As in r4, Fgf signaling is also required for r5/r6 gene expression, but again in a

different capacity. Specifically, disruption of Fgf signaling in zebrafish leads to the loss of all r5/r6 gene expression (except *hnf1ba*), while it only affects expression of Fgf signaling components in r4. Since *hnf1ba* expression persists upon Fgf disruption (and *vice versa*), *hnf1ba* or Fgf cannot be individually sufficient for r5/r6 gene expression. Indeed, several studies have demonstrated that both Fgf and *hnf1ba* are required to regulate expression of *MafB/valentino* in r5/r6 and *egr2*/*krox20* in r5 (Hernandez *et al.*, 2004; Wiellette and Sive, 2003). Hence, Fgf signaling acts as a subprogram in r4, but is an essential component of the r5/r6 regulatory network. Lastly, mutations in the PG3 *Hox* genes in the mouse affect formation of the abducens neurons, but do not affect rhombomere-specific gene expression – e.g. *egr2/krox20* expression in r5 is normal in PG3 mutants (Gaufo *et al.*, 2003). These analyses indicate that *Hox* genes function differently in r4 compared to r5/r6. In the former case, *hoxb1b* plays a key role along with RA, such that combined disruption of these factors prevents r4 specification. In contrast, the PG3 *Hox* genes have a more modest role in r5/r6, where their disruption affects neuronal differentiation, but not expression of most r5/r6 TFs.

The r4 regulatory network is very robust

From the previous discussion it emerges that *hoxb1b* and RA are jointly required for r4 formation, while multiple factors (at least *hnf1ba, MafB/valentino*, *egr2/krox20,* RA and Fgf) are individually required for formation of r5/r6. Since disruption of these factors leads a failure of proper rhombomere formation, we consider them to represent key factors for development of the corresponding rhombomere. It is striking that each of these genes was identified more than ten years ago and this raises the questions why additional key factors have not been identified since. As discussed, the forward genetic approaches may have missed such genes because the assays used for screening were by necessity relatively broad-based and also did not reach saturation. However, it is also possible that these are the only key factors in each rhombomere and that the rest of the genes acting in these networks are not essential. With the help of straightforward ZFN, TALEN and CRISPR/Cas9 approaches, a number of genes from the r4 and r5/r6 gene networks have now been mutated in zebrafish and the resulting animals assayed for hindbrain defects. Strikingly, of eight recently reported mutants, (*gas6, sall4, egfl6, celf2, greb1l, dusp2, dusp6* and *gbx1*), none show disruption of r4 or r5/r6 gene expression (Maurer and Sagerström, 2018; Ghosh *et al.*, 2018). Future analyses will be required to determine if double mutants produce phenotypes – as was the case for simultaneous disruption of *hoxb1b* and RA signaling – or if the regulatory networks are resistant also to such situations. While many more mutants remain to be analyzed before the r4 and r5/r6 gene sets are fully tested, at this stage it appears that these networks – particularly the r4 network – are highly redundant with only a few key genes being absolutely required for their integrity.

An updated model for the r4 and r5/r6 gene regulatory networks

With the addition of this recent gene expression and functional data, a revised model for the regulatory networks controlling zebrafish r4 and r5/r6 formation is emerging (Fig. 3). In r4, RA acts together with *hoxb1b* to control expression of all r4 genes tested to date. Furthermore, Fgf signaling is required for expression of a subset of r4 genes – specifically those that act in the Fgf signaling cascade. Strikingly, mutational analyses indicate that no single gene is absolutely required for r4 formation. Instead, some genes are required for limited aspects of the r4 fate (e.g. *hoxb1a* is required for differentiation of Mauthner neurons, but not for other aspects of r4 formation), while most r4 genes produce no discernible phenotype when mutated (e.g. *egfl6*). Formation of r5/r6 appears to be controlled in a different manner. Specifically, there are at least five factors (RA, Fgf, *hnf1b*, *MafB/valentino* and *egr2/krox20*), disruptions of which leads to extensive loss of r5/r6 fates, while a few other genes have more limited roles (e.g. PG3 *Hox* genes) and yet others produce no phenotypes when mutated (e.g. *gas6*). Hence, r4 and r5/r6 formation differs in key aspects. First, gene function in r4 appears completely redundant (at least in terms of the genes analyzed to date), while r5/r6 expresses several genes that are individually absolutely required for r5/r6 formation. It is not clear why the r4 network would be more robust, but r4 is the first rhombomere to form and it plays a key role as a Fgf signaling center during hindbrain development. Perhaps this situation provided sufficient evolutionary pressure to ensure that r4 can form even when individual components of the r4 regulatory network are disrupted. Second, *Hox* genes have different roles in r4 relative to r5/r6. In particular, *hoxb1b* occupies a key point together with RA in r4. In contrast, PG3 *Hox* genes appear required only for neuronal differentiation. In this regard, *hoxb1a* (which is required for differentiation of Mauthner neurons) acts more similarly to the PG3 *Hox* genes in r5/r6 than to the closely related *hoxb1b* gene in r4. Since much of the recent work discussed in this review was done in zebrafish, it is possible that some aspects of the updated model (Fig. 3) represent zebrafish-specific features. Indeed, we recently reported a comparison of PG1 *Hox* function in zebrafish versus mouse and identified some species-specific roles for these genes (Weicksel *et al.*, 2014). However, those species-specific effects were relatively subtle, leading us to hypothesize that most of the features of the revised model in Fig. 3 will be representative of

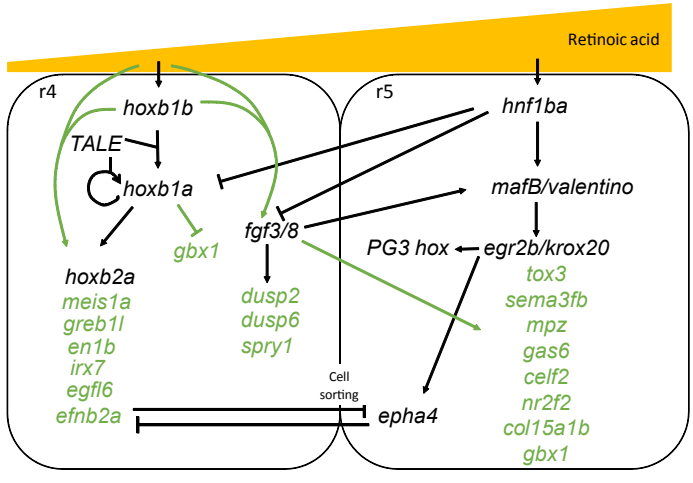

Fig. 3. An updated model of regulatory networks controlling r4 (left) and r5 (right) formation. *The diagram is based on zebrafish data and relevant comparisons to the mouse are made in the text. Arrows and genes colored in green represent recently demonstrated interactions that expand on the model shown in Fig. 2.*

both fish and mouse.

It is likely that additional genes are required for r4 and r5/r6 formation. For instance, while *hoxb1a* mutants retain expression of all r4 genes tested so far, these animals nevertheless lack Mauthner neurons. This suggests that there are additional genes required for Mauthner formation that are *hoxb1a*-dependent, but these have yet to be identified. Furthermore, animals mutant for other r4 genes do not lack Mauthner neurons, suggesting that *hoxb1a* may define a Mauthner differentiation pathway that is separate from the rest of the r4 regulatory network. Similarly, loss of PG3 *Hox* genes affects the formation of abducens neurons, but it does not affect expression of most r5/r6 genes – again indicating that there may be a PG3 *Hox*-regulated pathway within r5/r6 that controls neuronal differentiation. With the continued rapid accumulation of gene expression data and functional analyses in mutant animals, these regulatory networks should continue to become better defined in the near future.

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