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HOX gene cluster (de)regulation in brain: from neurodevelopment to malignant glial tumours

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Abstract:

HOX genes encode a family of evolutionarily conserved homeodomain transcription factors that are crucial both during development and adult life. In humans, 39 HOX genes are arranged in four clusters (HOXA, B, C, and D) in chromosomes 7, 17, 12 and 2, respectively. During embryonic development, particular epigenetic states accompany their expression along the anterior-posterior body axis. This tightly regulated temporal-spatial expression pattern reflects their relative chromosomal localization, and is critical for normal embryonic brain development, when HOX genes are mainly expressed in the hindbrain and mostly absent in the forebrain region. Epigenetic marks, mostly polycomb-associated, are dynamically regulated at HOX loci and regulatory regions to ensure the finely tuned HOX activation and repression, highlighting a crucial epigenetic plasticity necessary for homeostatic development. HOX genes are essentially absent in healthy adult brain, whereas they are detected in malignant brain tumours, namely gliomas, where HOX genes display critical roles by regulating several hallmarks of cancer. Here, we review the major mechanisms involved in HOX genes (de)regulation in the brain, from embryonic to adult stages, in physiological and oncologic conditions. We focus particularly on the emerging causes of HOX gene deregulation in glioma, as well as on their functional and clinical implications.

Keywords: glioma; epigenetics; transcriptional regulation; neurodevelopment; homeobox

Abbreviations: 3C: chromosome conformation capture; 3D: 3-dimensional; 4C-seq: circularized chromosome conformation capture with deep sequencing; 5C: chromosome conformation capture carbon copy; AQB: AC1Q3QWB drug; CAM: chicken chorioallantoic membrane; CGGA: Chinese Glioma Genome Atlas; ChIP: chromatin immunoprecipitation; CHROMO: chromatin organization modifier; CNS: central nervous system; CNV: copy number variations; COMPASS: complex proteins associated with Set1; DNA: deoxyribonucleic acid; ESCs: embryonic stem cells; GBM: glioblastoma; G-CIMP: glioma CpG island methylator phenotype; GSCs: GBM stem cells; H2AK119ub: ubiquitination of H2AK119 residues; H3K27ac: histone H3 lysine 27 acetylation; H3K27me3: histone H3 lysine 27 trimethylation; H3K4me3: histone H3 lysine 4 trimethylation; Hi-C: chromosome capture followed by high-throughput sequencing; HOX: homeobox; HUVEC: human umbilical vein endothelial cells; LGG: low-grade gliomas; lncRNA: long non-coding RNA; miRNAs: micro RNA; mRNA: messenger RNA; OS: overall survival; PARs: promoter-associated RNAs; PcG: polycomb group; PCR: polymerase chain reaction; PDX: patient-derived xenograft; PRE: polycomb responsive element; RA: retinoic acid; RNA: ribonucleic acid; SINE: short interspersed nuclear elements; TAD: topological associating domain; TCGA: The Cancer Genome Atlas; TMZ:

temozolomide; TrxG: trithorax-group proteins; TSS: transcription start sites; WHO: World Health Organization

1. Introduction

Genetic mutations in the fruit fly *Drosophila melanogaster* that resulted in homeotic transformations of one body segment to another (e.g., antennae growing at the posterior part instead of at the head of the fly) led to the identification of master transcriptional regulators named homeobox genes [1-5]. These genes are characterized by a consensus DNA sequence of 183 base pairs, the so-called homeobox, that encodes the homeodomain, a 61-amino acid peptide motif with a distinctive helix-loop-helix-turn-helix structure [6]. This homeodomain serves, among other functions, as a DNA binding domain that preferentially recognizes a specific TA-rich core DNA sequence, such as TAAT or TTAT [7-10]. Homeobox genes are of paramount importance for the developmental and post-developmental regulation of morphogenesis, patterning and differentiation, independently of the organ, limb or tissue where they are expressed [11-19].

HOX genes represent the main subset of the homeobox family. They display evolutionarily conserved transcription factor functions common to all bilaterian animals [20], and are considered crucial for the establishment of segmental identity along the anterior-posterior body axis of vertebrates. HOX proteins also have non-transcriptional activities and are implicated in regulating various processes, such as DNA replication and repair, mRNA translation, and protein degradation (reviewed by Rezsohazy et al. [21]). Structurally, they are divided into two exons and one intron, being the homeobox sequence present in the second exon (Fig. 1A). In *D. melanogaster*, eight collinear HOX genes, split into two clusters (the antennapedia and bithorax complexes), are responsible for segmental patterning. In amniotes, there are 39 HOX genes distributed into four clusters (HOXA, HOXB, HOXC, and HOXD) according to their chromosomal localization (7p15, 17q21.2, 12q13, and 2q31, respectively, in humans) [22]. Each cluster is divided into 13 paralogue groups (HOX1 at the 3' end to HOX13 at the 5' end of each cluster), with 9 to 11 genes assigned to each cluster, based on their sequence homology and position within the cluster (Fig. 1B). It is thought that these four clusters originated during vertebrate evolution due to two rounds of whole genome duplication that resulted in paralogue clusters with partial redundant functions [20,23,24]. The temporal-spatial collinear expression of HOX genes during embryogenesis [25,23] is one of the most organized and captivating mechanisms of gene regulation, in which the expression pattern of each paralogue group directly mirrors their collinear chromosomal organization. The first paralogues (HOX1 and HOX2) are the first to be expressed (shortly after the establishment of the primitive streak), followed by the other paralogues (from the 3' end to the 5' end in each cluster) in a time/developmental stage-dependent manner. In addition to their temporally regulated expression, they are gradually expressed from more anterior regions to more posterior regions of the embryo. This spatial collinearity (i.e., the relation between the relative chromosomal position of a HOX gene and its spatial expression in the body) was

first described in *D. melanogaster* [4]. Since then, it has been observed throughout the animal kingdom [26]. Nonetheless, temporal collinearity is reserved to bilaterians with intact chromosomal organization of HOX in clusters [27,28]. Unsurprisingly, at least 15 genetic disorders were associated with germline mutations in 10 out of 39 HOX genes in humans (as reviewed in Quinonez, Innis [29]). In mouse, loss-of-function mutations of 26 out of 28 tested Hox genes resulted in a particular phenotype/defect in the animal, which mirrors those observed in humans and may thus help clinicians to better predict the effects of alterations in specific HOX genes in patients [29].

This review will critically summarize the major molecular mechanisms of transcriptional regulation of HOX genes in the healthy brain, with a special focus on their dynamic epigenetic landscapes during critical stages of neuro-development. In addition, it will discuss the most critical molecular alterations and functional roles of HOX genes in brain tumours, as well as their recently highlighted clinical relevance in patients.

2. General principles of HOX regulation

The collinear activation, in time and space, of HOX genes during vertebrate development relies on a multi-scale regulation that involves the *cis*-regulatory landscape, the three-dimensional (3D) chromatin configuration, the histone modification pattern and RNA-based regulation [27].

- Proximal and remote *cis*-regulatory regions

Early studies in transgenic mice showed that some individual Hox genes inserted at ectopic positions in the genome can recapitulate their endogenous expression pattern, indicating that the necessary regulatory sequences are in their vicinity [30,31]. Accordingly, multiple proximal *cis*-regulatory sequences that can influence transcription of neighbouring genes were identified within Hox clusters [32-34]. For instance, two retinoic acid (RA) response elements embedded in the Hoxb cluster control expression of *Hoxb5* to *Hoxb9* in the developing neural tube [35]. Such proximal regulatory sequences provide a frame for the evolutionary conserved temporal collinear activation of HOX genes. However, novel HOX-associated patterning functions, which often use the collinear property of only a subset of genes from the same cluster, emerged in vertebrate lineages. These novel functions often rely on *cis*-regulatory sequences located outside the HOX cluster. For instance the development of both proximal and distal limb segments, in the limb buds, relies on two subsequent waves of Hoxd genes transcription, controlled by distinct remote *cis*-regulatory regions located on each side of the gene cluster [36-38]. A similar long-range regulation is also documented at the other HOX clusters to control the coordinated activation of HOX genes in specific developmental stages and tissues [41-43]. These observations revealed the importance of the remote transcriptional control to the HOX-

mediated patterning of the developing vertebrate embryo. In addition, as the distant enhancers and HOX promoters must physically interact, the 3D chromatin conformation has to be taken into account to understand HOX regulation. Accordingly, a significant advance in our understanding of HOX control came with the development of molecular tools to assess the 3D genome organization at high-resolution.

- A 3D chromatin conformation-based regulation

The development of the Chromosome Conformation Capture (3C) methodology and its derivatives (circularized chromosome conformation capture with deep sequencing, 4C-seq; chromosome conformation capture carbon copy, 5C; and chromosome capture followed by high-throughput sequencing, Hi-C) [44] allowed the analysis of the relationship between genome architecture and gene expression control over time. Specifically, Hi-C approaches showed that chromosomes are divided into mega base-scale regions, called Topological associating domains (TADs) in which chromatin interactions are highly favoured [45,46]. These domains are relatively stable in the different cell types, and are proposed to be structural features of the genome [45]. Strikingly, both HOXA and HOXD clusters lie at the boundary between two TADs [45]. This topological organization might separate these compact gene clusters in two independent functional units that will specify contacts between remote enhancers and their cognate HOX target genes during development (Fig. 2). This scheme explains the bi-modal regulation of the Hoxd cluster during limb development. Long-range interactions between the 3' Hoxd genes and enhancers located in the telomeric gene desert occur within the 3' (telomeric) TAD. Conversely, 5' genes segregate in the 5' (centromeric) TAD where they contact enhancers in the centromeric gene desert. Interestingly, a subset of contacts between Hox promoters and their distal enhancers occur by default within the TAD, regardless of the enhancer activity and their expression status [39,41]. In parallel to the enhancer-promoter contacts established concomitantly with gene activation [48,49], this pre-established configuration at a subset of genes might prime them for rapid induction [32,50].

The molecular bases of TAD ontogeny at HOX cluster are not fully understood. A critical factor is the chromatin barrier insulator CTCF (CCCTC-binding factor) that is often located at TAD boundaries [45]. Specifically, deletion of CTCF binding sites within the Hoxa cluster can reposition the TAD boundary and lead to misregulation of Hox genes expression [51,52]. However, other yet to be identified actors might contribute to TAD boundary positioning [53]. These include the structural protein cohesin that promotes DNA loop formation between distant regions [54], as well as genomic features, such as the presence of short interspersed nuclear elements (SINE) [45]. Tissue-specific transcription factors also could influence TAD boundary position. This hypothesis is supported by

the observation that this position can vary in a tissue-specific manner at the HOXA and HOXD clusters (reviewed in [32]), with reallocation of some genes from one TAD to the other. Therefore, there could be an interplay between intra-cluster TAD boundaries and tissue-specific transcriptional machinery [32].

Unlike HOXA and HOXD, the HOXB and HOXC clusters are not associated with a TAD boundary [45], suggesting that TADs are not required for long-range regulations within these clusters. Their genomic organization might create a physical separation between the 3' and 5' parts of each cluster. Indeed, due to its remote localization, *HOXB13* is isolated from *HOXB1-HOXB9,* while the HOXC cluster does not contain paralogues for the groups 1-3 (Fig. 1B).

- HOX genes are *bona-fide* polycomb targets

HOX gene activation is associated with dynamic changes in their chromatin signature. Hence, expression patterns induced by productive enhancer-promoter contacts are maintained and faithfully transmitted to daughter cells. Besides the gain of the activating H3K27ac (acetylation of histone H3 lysine 27) mark within enhancers [55], the most drastic changes affect the distribution of the repressive H3K27me3 and the permissive H3K4me3 marks within HOX clusters.

In embryonic stem cells (ESCs), which do not express HOX genes, whole HOX clusters are fully decorated by H3K27me3, while at their promoter area this mark co-exists with H3K4me3, constituting the so-called bivalent chromatin [56,57] (Fig. 3A). It has been suggested that bivalent chromatin domains repress transcription of genes through H3K27me3, while keeping them 'poised' for alternative fates, through resolution into either H3K4me3 or H3K27me3, upon stem cell differentiation [56]. In the developing embryo, collinear activation of HOX genes is accompanied by a progressive loss of H3K27me3 associated with H3K4me3 gain. Consequently, H3K27me3- and H3K4me3-marked domains demarcate repressed and active HOX genes, respectively, along the cluster (Fig. 3B). In differentiated cells and tissues where all HOX genes are repressed, such as the forebrain, H3K27me3 coats the whole clusters [58,59,57] (Fig. 3C). This observation highlights the importance of the polycomb (PcG) and trithorax (TrxG) groups of proteins that regulate H3K27me3 and H3K4me3 deposition, respectively, in the epigenetic regulation of HOX genes.

PcG-mediated silencing relies on two complexes that act sequentially: Polycomb Repressive complex 1 and 2 (PRC1 and PRC2). In the canonical model, PRC2 first deposits H3K27me3 at the targeted chromatin. H3K27me3 then recruits PRC1 that induces a compacted chromatin state mainly through ubiquitination of H2AK119 residues (H2AK119ub). This two-step model is supported by the finding that while both complexes are required for proper HOX gene silencing [60,58,61], PRC1 deficiency

does not affect the PRC2-mediated H3K27me3 pattern [58]. Unlike in *Drosophila*, no polycomb responsive element (PRE) has been formally described in vertebrates, questioning the mechanism for PRC targeting at specific loci. The observation that PRC2 components can associate with long non-coding RNAs (lncRNA) and promoter-associated RNAs (PARs) suggests the implication of RNA transcripts in this process [62-64]. For instance, the lncRNA HOTAIR, which is produced in the 5' part of the HOXC cluster, recruits PRC2 and acts in *trans* to silence the genes located in the 5' part of the HOXD cluster in cultured cells [63,65]. Similarly, it has been suggested that Hoxa adjacent lncRNA 1 (*Harl1*), a lncRNA located approximatively 50Kb downstream of the HOXA cluster, represses this cluster in mouse ESCs by recruiting PRC2 to Hoxa promoters [66]. However, functional studies in animals indicate that *Harl1* is dispensable for normal development [67,68], and brought conflicting results on *Hotair* [69-71], questioning the role of lncRNAs in the regulation of HOX clusters *in vivo*. It has also been proposed that PRC2 recruitment could be responsive to permissive chromatin signatures at promoters [72]. In agreement, studies in mammalian cells and tissues showed that PRC2 is recruited by default at transcriptionally inactive CpG islands/promoters [73,74].

TrxG proteins are the second main epigenetic regulator of HOX clusters. Specifically, deposition of H3K4me3 at HOX clusters in mammals relies on the COMPASS-like complex that contains MLL1 and 2, the homologues of *Drosophila* Trx [75]. Interestingly, this complex also contains the H3K27me3-demethylase KDM6A that removes H3K27me3 at HOX loci [76-78]. This suggests that H3K4me3 deposition and H3K27me3 removal are coupled at HOX promoters. Like for PRC2, it has been suggested that lncRNAs promote recruitment of the TrxG/MLL complex. For instance, *HOTTIP*, located at the 5' tip of the HOXA locus, recruits in *cis* the WDR5/MLL proteins and promotes H3K4me3 deposition [79]. Alternatively, as observed for other COMPASS-like complexes [80], TrxG/MLL complexes could be recruited at promoters through association with the transcriptional machinery.

- Active and repressed HOX genes are within distinct nuclear compartments in the nucleus

Altogether, a model emerges whereby H3K27me3 is deposited by default at transcriptionally inactive HOX genes, a process that can be promoted by lncRNA activity. Upon enhancer-induced activation, the transcriptional machinery would bring TrxG/MLL/KDM6A complexes to promoters to sustain HOX gene transcriptional activity by inducing H3K4me3 deposition and H3K27me3 removal. Interestingly, the dynamics of H3K27me3/H3K4me3 distribution along the different HOX clusters impacts their 3D architecture. Indeed, 4C approaches showed the segregation of active and repressed HOX genes within distinct nuclear compartments [55,59]. Specifically, H3K27me3-marked HOX

loci cluster together within inactive nuclear compartments, denoted polycomb bodies, where they can contact, although at a lower frequency, other polycomb targets through intra- and inter-chromosomal interactions [+ Pirotta & Li, 81,82]. Upon induction, HOX genes switch to more discrete H3K4me3-associated compartments, thus modifying the cluster 3D organization [55,59]. This spatial separation between the silent and expressed parts of HOX clusters might facilitate their finely tuned regulation.

- miRNA and post-transcriptional regulation

In addition to transcriptional regulation, HOX expression is also controlled at the post-transcriptional level by the mean of several processes, including polycistronic transcription, RNA processing or sequence-specific transcriptional control. These issues have been recently extensively reviewed [Casaca et al., 2018]. Among those, one important mechanism is regulation via microRNA (miRNA) repression. These short RNA species negatively regulate gene expression by binding target mRNA in a sequence-specific manner, further leading to degradation *via* the RNA-induced silencing complex (reviewed in Bartel, 2018). Several highly conserved miRNA families are embedded within HOX clusters, including for instance the miR-10, miR-615 and miR-196 families. Interestingly, among the predicted targets of these HOX-embedded miRNAs, many are protein-coding HOX mRNAs [Yekta et al., 2008]. To which extent these miRNAs indeed contribute to control and coordinate HOX output remain to be fully determined. Nonetheless, functional studies have highlighted that *miR-196* and *miR-10* do contribute to HOX mRNA regulation in vertebrates [Mandsfiled and McGlinn, 2012; Casaca, 2018]. This is best illustrated by miR-196 that acts by delimiting *Hoxb8* activity in developing hind limb and neural tissue [Hornstein , 2005; Asli and Kessel, 2010].

3. Roles of HOX genes in the developing brain

Most HOX genes are expressed in the developing vertebrate central nervous system (CNS) where they have critical functions. They first play a central role in cell fate determination within hindbrain and spinal cord segments, further contributing to the establishment of functional neuronal networks [86; 98]. These structures are indeed key coordination centres of the CNS that, via dedicated neural assemblies, regulate complex physiological processes, including breathing, locomotion, heartbeat, and different sensory systems [107].

Following the onset of neural induction, the early vertebrate hindbrain is transiently segmented in seven or eight cellular compartments, depending on the species, called rhombomeres (r1 to r8) (Fig. 1B). While no physical barrier exists between them, cells of each rhombomere remain well segregated and do not mix with those of the other rhombomeres [83,84]. Each of these segments constitutes an independent lineage-restricted unit that creates regional diversity along the anterior-posterior axis of

the developing hindbrain, thus acting as a template for the future adult brainstem structure and function. Each rhombomere is the source of distinctive neural progenitors that ultimately generate rhombomere-specific populations of neurons [85]. For instance, among motor neurons, trigeminal neurons develop in r2/r3, while facial and vagal neurons form in r4 and r7/r8, respectively. Although such a clear segmentation is not present in the spinal cord, neuronal regional diversity is also observed along the anterior-posterior axis. Specifically, spinal motor neurons are organized into longitudinal columns along the four main spinal cord units: cervical, thoracic, lumbar and sacral [86]. For instance, phrenic motor neurons are generated at the anterior cervical level, while preganglionic and hypaxial neurons form at the thoracic level (Fig. 1B).

Strikingly, specific combinations of Hox gene expression and/or expression levels define the regional identity in hindbrain and spinal cord. Specifically, Hox1-Hox5 paralogue group genes display rhombomere-specific nested expression patterns in hindbrain. Similarly, Hox4-Hox11 genes are expressed in a nested fashion along the anterior-posterior spinal cord axis [87,88,86,89] (Fig. 1B). Disturbance of these patterns in the mouse and other vertebrate models have revealed that HOX genes play a key role in the segmentation and specification of several rhombomeres in hindbrain, and in the maintenance of columnar identity in spinal cord [90-93,86].

The HOX combinatorial code in hindbrain and spinal cord is also involved in key steps of neural development by acting as a determinant to control the formation of distinct neuronal subtypes within the different segments. Specifically, studies conducted on motor neuron specification highlighted the importance of HOX transcription factors in regulating the diversity and identity of motor neuron pools in spinal cord, and in establishing the pattern and specificity of limb muscle innervation [94,90,95,92,86,96]. In addition, studies conducted in Hox conditional mouse mutants support a role for Hox genes in later stages of CNS development to generate neural circuit assemblies. For instance, the formation of respiratory, somatosensory and auditory circuits is perturbed in the absence of specific Hox genes [97-99].

Therefore, neuronal subtype specification and connectivity in hindbrain and spinal cord rely on HOX-specific expression patterns and the associated regionalization along the anterior-posterior axis. These expression patterns are set up sequentially during two phases of regulation in progenitors and post-mitotic neurons, respectively. They involve a complex interplay between morphogen signals, cross-regulatory interactions between Hox genes, and polycomb-associated epigenetic modifications. First, in the neural tube, opposite gradients of RA and Fibroblast growth factor (FGF) signalling act in concert to establish the initial Hox expression pattern in the early CNS [100,18]. RA primarily promotes the expression of the Hox1-5 paralogue group of genes through activation and direct binding

of RA receptors (RARs) [101]. In spinal cord, FGF establishes the Hox4-Hox10 gene expression patterns through the induction and subsequent binding of the homeodomain protein factors CDX [102,103]. Studies in differentiating mouse neural progenitors showed that binding of RARs and CDX to their cognate Hox target genes and their subsequent activation are associated with the rapid removal of the repressive H3K27me3 mark [104]. This chromatin remodelling provides a template to transmit these initial gene expression patterns to early post-mitotic neurons where they will be refined and segmentally restricted. Although neural progenitors display a nested pattern of Hox expression, clear Hox posterior boundaries will be indeed established at the time of differentiation and often maintained up to late foetal stages in restricted neuronal subpopulations. This second regulatory phase relies on direct self- and cross-regulatory interactions between HOX transcriptions factors and Hox genes, associated with the combined action of other transcription factors [105,106]. For instance, a complex interplay between the KROX20 transcription factor and the Hox1 and Hox2 paralogue groups ensures that *Hoxb1* expression is restricted to r4 in the vertebrate hindbrain, where it will be then maintained through an auto-regulatory loop [107,108]. EZH2, the enzymatic component of PRC2, is required to maintain Hox expression patterns in the segmented hindbrain. Accordingly, its neural-specific depletion in the mouse leads to migration and connectivity defects of pre-cerebellar pontine neurons [109]. In the spinal cord, PRC2-mediated H3K27me3 pattern at Hox loci is determined during the progenitor phase. However, Hox gene expression pattern is refined and maintained in post-mitotic cells through a mechanism that seems to depend on PRC1, the other PRC complex. Specifically, depletion of the PRC1 component BMI-1 affects *Hoxc9* expression and the spinal cord columnar identity [110].

After neurulation, HOX expression patterns are maintained up to late foetal/early post-natal stages in restricted neuronal subpopulation of the hindbrain, and in restricted domains of the spinal cord [95,98,99, Gofflot and Lizen, 2018]. A handful of studies further report that HOX expression may persist post-natally and in the adult brain. For instance, RT-PCR analyses in human adult brain extract highlighted the expression of the 3' HOX genes (HOX1-HOX7 paralogues) [12]. A more systematic analysis conducted in the adult mouse brain evidenced that the expression of genes belonging to Hox2-Hox8 paraloguegroups is maintained in the hindbrain derivatives. The same analysis also support that some Hox transcripts are neo-expressed in specific brain regions at adulthood [Hutlet et al., 2014]. These observations suggest that HOX transcripts could be functionally required in the brain after birth, stressing the need to formally assess their function at adulthood. Indeed, several lines of evidence mainly based on functional *in vivo* data obtained for the *Hoxa2* and *Hoxa5* loci suggest a role in synapse formation and maturation [reviewed in Gofflot and Lizen, 2018]. Unlike in hindbrain and spinal cord, HOX genes are not expressed in the developing forebrain, the most anterior part of

the developing CNS, and also in adult brain [111]. This repression is mediated by H3K27me3 [59], and is critical for brain expansion. This evolutionary conserved feature of the CNS leads to a more prominent anterior region (brain) compared with the posterior ones. A study conducted in *Drosophila* and the mouse revealed that brain expansion is severely reduced following Hox ectopic expression, or loss of PcG function [112]. Surprisingly, mutation of the *Abd-B* HOX gene rescues the reduced brain proliferation observed in PRC2 mutants in *Drosophila*. This intriguing observation, which has not yet been assessed in vertebrate brain, suggests that in the developing *Drosophila* brain, one of the primary roles of PRC2 is to repress Hox genes [113]. From this analysis, it emerges a picture whereby HOX genes repress the neural stem cell proliferation programme in the more posterior parts. PcG-mediated repression of HOX genes prevent this anti-proliferative action in the forebrain, thus promoting brain expansion [113]. Altogether, this observation stresses the importance of maintaining HOX genes repressed in forebrain.

4. HOX deregulation in brain gliomas

HOX genes may present tumour suppressive or oncogenic functions, and their expression is frequently altered in cancer, including leukaemia [114-118], breast [119-121], brain [122-127], lung [128,129], colon [130], cervix [131], bladder [132,133], and kidney cancers [134]. In the context of this review, we will focus on brain tumours, specifically high-grade gliomas.

4.1. Malignant primary brain tumours

Malignant primary brain tumours represent approximately 32% of all brain tumours and affect more than 250 000 individuals each year worldwide (17th most common cancer type) [135]. Despite their relatively low incidence compared with other primary cancers (e.g., lung, breast, prostate and colorectal cancers) and metastatic brain tumours, they constitute a major source of morbidity and mortality. They are the 12th most deadly cancer worldwide (almost 190 000 estimated deaths per year) [135], and rank first in average of years of life lost among all tumour types [136].

Gliomas represent the majority of all malignant brain tumours (81%).

Until 2016, gliomas were traditionally classified based on histologic features as astrocytic, oligodendroglial, oligoastrocytic (mixed), or ependymal tumours, as described in the 2007 World Health Organization (WHO) classification guidelines [139]. In this classification, tumours were named after the normal cells from which they resembled. However, this classification methodology was prone to considerable inter-observer variability, particularly in the context of diffusely infiltrating gliomas (e.g., differences in the classification of astrocytoma vs. oligodendroglioma vs. oligoastrocytoma) [140-142]. Moreover, advances in (epi)genetics and transcriptomic analyses have

shed light on the biological and clinical variability observed within each histologically-defined glioma entity [143,142]. These observations suggested that some of the molecular alterations underlying this variability might in fact be used as biomarkers for more accurate glioma classification [143], which contributed to the 2016 revision of the guidelines [141]. For example, according to the most recent WHO classification of CNS tumours, gliomas can be isocitrate dehydrogenase (IDH)-wildtype or IDH-mutant [142]. Gliomas are also stratified according to their malignancy grade, from benign grade I to highly-malignant grade IV. Glioblastoma (GBM) is the most common and malignant form of glioma [137], being almost universally fatal with very low 2- and 5-year survival rates [138]. IDH-wildtype GBMs are the most common (90%), occur mainly in elderly patients (median age at diagnosis: 62 years), are localized in the supratentorial region, and correspond to primary GBM that develop *de novo* (i.e., with a short clinical history of less than 3 months before diagnosis, and without a pre-existing lower-grade precursor lesion) [143,142]. They are associated with copy number gains on chromosome 7 (where the HOXA cluster is located), chromosome 10 monosomy, *EGFR* amplification, and mutations in *PTEN*, *TERT*, *CDKN2A* and *CDKN2B* genes. *TERT* promoter mutation is mutually exclusive with *ATRX* mutation, which are common in IDH-mutant GBM [142]. IDH-mutant GBMs typically occur in younger adults (median age at diagnosis: 44 years), are preferentially localized in the frontal lobe, and include most of secondary GBMs (i.e., GBMs that develop from a pre-existing diffuse or anaplastic astrocytoma) [142,143]. These tumours are characterized by copy number gains on chromosome 7, loss of heterozygosity of the 17p arm, *TP53* and *ATRX* mutations, and the glioma CpG island methylator phenotype (G-CIMP) [142,143].

4.2. Aberrant HOX gene expression in glioma

Abdel-Fattah et al. [125] were the first to report the aberrant overexpression of 9 of the 39 HOX genes (*HOXA6*, *A7*, *A9*, *A13*, *B13*, *D4*, *D9*, *D10*, and *D13*) in gliomas compared with normal human astrocytes and temporal lobe cells (these may include astrocytes, microglia, and neurons; summarized in Table 1). In 2008, Murat et al. [124] described the overexpression of several HOX genes (*HOXA2*, *A3*, *A5*, *A7*, *A9*, *A10*, *C6*, *D4*, *D8*, and *D10)* in tumour samples compared with non-neoplastic brain samples from patients with GBM. In 2009, Buccoliero et al. [144] analysed the expression of HOXD genes in 14 paediatric grade I gliomas and 6 non-neoplastic human brain tissues, and described that *HOXD1* and *HOXD12* were overexpressed in grade I gliomas compared to non-neoplastic tissues, while *HOXD3*, *D8*, *D9*, and *D10* presented lower expression in grade I glioma (the last three were exceptionally expressed in non-neoplastic tissues). On the other hand, *HOXD4*, *D11*, and *D13* were not expressed in grade I glioma. Sun et al. [145] reported later that HOXD10 was expressed in GBM. In 2010, Costa et al. [122] showed widespread activation of HOX genes (*HOXA1*, *A2*, *A3*, *A4*, *A5*, *A7*, *A9*, *A10*, *B7*, and *C6*) in a subset of GBM patients compared with less malignant gliomas or

normal brain tissues. Duan et al. [146] later corroborated these findings by describing that HOXA9, A11, and A13 proteins, and *HOXA9*, *A10*, *A11*, and *A13* mRNA levels were strongly upregulated in GBM compared with grade II/III gliomas. However, *HOXA11* expression was significantly downregulated in recurrent GBMs compared with primary GBMs [147]. Several other studies also reported the overexpression of specific HOX genes in GBM compared with non-neoplastic brain samples: *HOXA1* [148], *HOXA9* [150], *HOXA13* [146], *HOXB3* [151], *HOXB7* [152], *HOXB9* [153], *HOXC6* [154], *HOXC9* [155], *HOXC10* [156,157], *HOXD4* [158], and *HOXD9* [126]. Among these genes, *HOXA1*, *HOXA9*, *HOXA13*, *HOXB9*, and *HOXC10* were expressed in a grade-dependent manner [148,150,146,153,156]. *HOXB1* and *HOXD10* expression levels were shown to be lower in glioma than in non-neoplastic samples [159,160], and *HOXB1* particularly low in GBM compared to grade I and II gliomas. Moreover, Gaspar et al. [123] found that *HOXA2*, *A5*, *A7*, and *A9* were overexpressed in short-term (<1 year) paediatric GBM survivors compared with long-term survivors. Gallo et al. [161] identified a specific gene signature of patient-derived GBM stem cells (GSCs), compared with normal human foetal neural stem cells and non-neoplastic brain cortical tissues, which included 24 of the 39 HOX genes. In agreement, Li et al. [162] reported that *CD133* expression, a marker of GBM stem cells, correlates with *HOXA5*, *A7*, *A10*, *C4*, and *C6* expression in GBM.

In summary, different studies reported that the expression of several HOX genes is consistently altered in glioma. The few discrepancies among studies, in which a particular HOX gene was described to be up- and down-regulated, might be explained by: i) the different methods used to evaluate gene/protein expression; ii) the different processing and origin of patients' samples; iii) the use of cell lines in some studies and tumour samples in others; and iv) the different types of samples used to define the "non-tumour" condition. Some of these limitations could be mitigated by using larger cohorts of equally processed and normalized samples (e.g. [163,164], n >500 glioma samples), and by defining standard methodologies for assessing gene expression alterations in these tumours.

4.3. HOX functional roles in GBM

Considering the aberrant expression of HOX genes in GBM, it is reasonable to hypothesize that they might be functionally relevant for the pathophysiology of these tumours. Various studies based on genetic manipulations of these genes, employing silencing or overexpressing approaches, reported that 18 of the 39 HOX genes, have important functional roles in glioma, including *HOXA5, A6, A7, A9, A10, A11, A13, B1, B3, B7, B9, B13, C6, C9, C10, D4, D9,* and *D10*. Most of the described roles are associated with oncogenic functions, although *HOXA11*, *HOXB1*, and *HOXD10* displaying tumour suppressive functions in GBM (summarized in Fig. 4).

<u>HOX genes act mostly as oncogenes in glioma</u>:

The genetic manipulation of *HOXA5*, *A9*, *A10*, *A13*, *B3*, *B7*, *B9*, *C6*, *C9*, *C10*, and *D9* showed that their expression increases the viability of GBM cell lines [151,154,152,149,146,153,161,157,156,150,126,165,122], and five of them (*HOXA9*, *A13*, *C6*, *C10*, and *D9*) also reduce cell death [122,146,156,150,126,151]. *HOXA7*, *A9*, *B7*, *B9*, *C9*, and *C10* also increased GBM cell lines migration capacity [166,152,153,157,156,150,165], while *HOXA6*, *A13*, *B3*, and *B13* promoted increased invasion [166,152,153,157,167,156,150,151,165,146]. Interestingly, *HOXC6*, *C10*, and *D9* also increased GBM cell lines colony formation capacity [157,156,126,154], and *HOXA13*, *C6*, and *D9* prevented cell cycle arrest [146,126,154].

Additionally, *HOXA9* and *HOXB9* increased GBM stem cell capacity and increased expression of stem cell markers (e.g., NESTIN) *in vitro* [153,150]. Similarly, *HOXD9* was highly expressed in the "side population" of GBM cells, a population of cells identified by flow cytometry and associated with cancer stem cell features, and in GSCs compared with normal astrocytes and neural stem/progenitor cells [126]. Interestingly, *HOXC9* was one of the 16 genes upregulated in $CD133^{+}$, a glioma stem cell marker, but not in $CD133^{-}$ GBM cell lines, compared in both cases with non-neoplastic cells. Moreover, in this 16-gene list, *HOXC9* and *E2F2* were the only genes associated with increased glioma malignancy [155].

In another *in vitro* study, *HOXC9* was shown to decrease Beclin1-mediated autophagy through inhibition of *DAPK1* (death-associated protein kinase 1) [165]. Moreover, it has been reported that *HOXA9* and *HOXC10* increase tumour angiogenesis *in vitro* and *in vivo* [150,168]. Indeed, *HOXC10* overexpression enhances tube formation, migration, and proliferation of Human Umbilical Vein Endothelial Cells (HUVEC, a specific type of endothelial cells) and neovascularization in the Chicken Chorioallantoic Membrane (CAM) *in vivo* assay {Tan, 2018 #9161}.

Three genes from the HOXA cluster have also been reported to have an important role in resistance to therapy. Specifically, *HOXA9* and *HOXA10* have been associated with decreased sensitivity to the chemotherapy drug temozolomide (TMZ) in GBM patients, a finding that was further supported by a causal relationship in cell lines [169,124,150], and *HOXA5* also decreased sensitivity to radiotherapy *in vitro* and *in vivo* [149].

In vivo, three genes of the "nine paralogues", *HOXA9*, *HOXB9*, and *HOXC9*, were shown to promote oncogenesis, as shown by the increased tumour volumes in subcutaneous GBM mouse models [153,150,165]. In clinically more relevant orthotopic models (i.e., based on intracranial implantation of GBM cells in mice), *HOXA5*, *A9*, *A10*, *A13*, *C6*, and *C9* expression increased the GBM-associated death rate [146,161,150,165,154,149]. Particularly interesting in these orthotopic models was *HOXA9*

and *HOXA5*, which increased the resistance to TMZ-based chemotherapy and radiotherapy, respectively, as indicated by the lower overall survival (OS) of mice bearing tumours positive for these HOX genes, as compared to negative tumours [150]. Interestingly, *HOXA9* overexpression in non-neoplastic human astrocytes induced their transformation when intracranially implanted, as shown by tumour formation and tumour-related death in 70% of the tested mice. Moreover, histological examination of the tumours formed after intracranial injection showed the presence of characteristic hallmarks of malignant gliomas. Remarkably, this tumorigenic capacity is not observed when cells are implanted subcutaneously, which stresses the critical importance of the tumour microenvironment.

Although most HOX genes display pro-tumour functions, a few exceptions have been reported. HOXB1 has tumour suppressive functions in GBM, by decreasing cell viability and promoting apoptosis *in vitro* [159]. HOXD10 decreases the invasive potential of GBM cell lines [170,171], and HOXA11 increases the sensitivity of GBM cell lines to chemotherapy and radiotherapy [147].

The functional impact of approximately half of HOX genes in GBM has already been described, particularly concerning the HOXA cluster. This stronger emphasis in HOXA genes could be partly explained due to the fact that they are on chromosome 7, which is frequently amplified in GBM, although HOXA gene copy number alterations have not been consistently associated with its expression [122,150,149,161]. Future studies on the roles of the other HOX genes in glioma will be critical to provide a more complete picture of their implication in these tumours. Additionally, considering the enormous heterogeneity observed between different glioma tumours, studies in glioma subtypes other than GBM will be of critical importance. Moreover, some of the previous findings could be consolidated by using: i) more biologically relevant models based on gene silencing, instead of overexpression approaches in which gene expression may reach unrealistic levels; ii) more clinically relevant patient-derived primary cell cultures, as opposed to cell lines that have been established and cultured for many years and that no longer faithfully reflect the original tumour; iii) stem cell cultures in serum-free conditions (e.g., neurospheres) that could be particularly useful to understand whether HOX roles are more relevant in the glioma stem-cell subpopulation or in more differentiated cells; and iv) 3D cultures (e.g., organoids) that better mimic the tumour microenvironment, endogenous cell organization, and organ structures, which could help might be relevant to better understand the role of HOX genes in more complex contexts.

4.4. Molecular bases of HOX gene deregulation in glioma

It is now clear that in glioma, the HOX gene activation pattern does not follow the coordinated collinear expression observed during normal embryonic development (described above). This process

is complex, which in addition to the deregulation of the main processes controlling HOX expression in normal contexts (described above) also involves epigenetic alterations, gene copy number variations, and direct activation by other transcriptional complexes.

Copy number variations (CNV) at HOX loci:

A pathognomonic characteristic of GBM IDH-wildtype is chromosome 7 trisomy (considered an early event in gliomagenesis) and chromosome 10 monosomy. As the HOXA locus is on chromosome 7, chromosome 7 trisomy might increase HOXA gene expression in these patients. Indeed, a correlation was observed between chromosome 7 gain and *HOXA1*, *HOXA4* [172], and *HOXA5* [149] expression in GBM and IDH-wildtype patients. However, this is not true for all HOXA genes because no correlation was observed between CNV and expression of *HOXA2* [172], *HOXA9* [150], *HOXA10* [161], and *HOXA13* [172]. This issue has been less studied at the other HOX clusters. Nonetheless, it was recently shown that overexpression of eight HOX genes distributed over the HOXB, C and D clusters was CNV-independent in IDH-wildtype samples [172]. Altogether, these observations support the hypothesis that CNV, if present, are not the main driving force of HOX gene overexpression in aggressive glioma.

Protein- or miRNA-directed targeting of HOX genes: Several miRNAs have been shown to negatively regulate HOX genes in GBM. For example, *HOXB1* is a target of hsa-miR-3175 [148], and *HOXD10* is a target of hsa-miR-21 [162], hsa-miR-10b [134], and hsa-miR-23a [149,160] in GBM. Moreover, hsa_circ_0074362 (circular RNA) was shown to act as a hsa-mir-1236-3p sponge to promote *HOXB7* expression in GBM [141].

Protein-gene promoter interactions is another well-known mechanism governing HOX deregulation. Gallo et al. [161] described that MLL protein, which contributes to HOXA gene regulation in haematopoiesis and leukemic cells, directly interacts with the promoter of *HOXA10* in glioblastoma stem cells. HOXA10 protein then interacts with the promoter region of *HOXA7* and *HOXC10*, activating their transcription [161].

Epigenetic alterations

As depicted in section 2, Polycomb group proteins and the associated H3K27me3 mark are key players in the control of HOX expression during normal development. A large number of studies have highlighted that *bona-fide* polycomb target genes are also more likely to gain aberrant DNA methylation in cancer cells [Ohm et al. 2007; Deneberg et al. 2011; 176]. Thus, not surprisingly, aberrant DNA methylation is a well-documented signature at HOX loci in glioma.

This was first observed by Martinez et al. [174] who evaluated the DNA methylation profile of 1505 CpGs sites, covering 807 genes, in 87 GBM patient samples. They identified 25 genes that were

hypermethylated and 7 that were hypomethylated in at least 20% of samples. Among the 25 hypermethylated genes, 3 were HOXA genes: *HOXA11* (hypermethylated in 51% of GBM), *HOXA9* (in 44%), and *HOXA5* (in 20%). Genome-wide analyses confirmed that the four HOX clusters tend to be hypermethylated in GBM and IDH-wildtype glioma, when compared to non-tumour brain samples (Kurscheid et al. [175]. This reminds the observation that hypermethylation of homeobox gene promoters, including HOX genes, is emerging as a pan-cancer signature [176]. Interestingly, the level of DNA methylation alteration can vary between genes and patients. In 2012, Di Vinci et al. [177] analysed DNA methylation of *HOXA3*, *A7*, *A9*, and *A10* in 63 glioma and found patient-specific DNA methylation patterns. This finding was validated by Pojo et al. [150] for *HOXA9*. *HOXA3* was the gene with the highest DNA methylation levels in GBM, while *HOXA7*, *A9*, and *A10* presented lower levels [177].

Gain of expression associated with gain of methylation at HOX loci is not in line with the canonical inhibitory effect of DNA promoter methylation on gene transcription. Refined analyses that integrated DNA methylation and strand-oriented expression patterns in glioma patient samples [172] provided some explanations to this apparent paradox. Specifically, for several genes, ectopic expression was associated with CpG islands/promoters that gained methylation at their borders, while their transcription start sites (TSS) remained methylation-free. At other genes, extensive methylation of their main CpG islands/promoter was associated with the use of an alternative promoter. For instance, in IDH-wildtype glioma, *HOXC11* transcription initiated from an unmethylated alternative promoter located 5 kb upstream the canonical, aberrantly methylated promoter [172]. This picture becomes even more complex in the case of chromosome 7 gain. Kurscheid et al. [175] suggested that hypermethylation of the HOXA cluster could compensate the CNV at this cluster in GBM cells with low expression level (HOX-low), while key CpG sites located in the HOXA locus could escape this hypermethylation phenotype in HOX-high GBM cells. Specifically, they showed that a CpG island located at the *HOXA10* non-canonical promoter escape to hypermethylation in HOX-high GBM cells.

Court et al. [172] provided evidences that besides the DNA methylation gain, HOX clusters in IDH-wildtype glioma samples are characterized also by dramatic reduction of H3K27me3. Given the importance of this mark for HOX gene repression in normal brain [59] (as previously detailed in section 2), its loss might be crucial for their aberrant expression in aggressive glioma. A study by Costa et al. [122] suggests that alteration of the PI3K pathway could be involved in this process. They showed that PTEN-dependent activation of the PI3K pathway resulted in AKT-mediated phosphorylation of EZH2, the catalytic subunit of the PRC2 complex, that suppresses H3K27me3, leading to derepression of previously silenced HOXA genes. Moreover, they demonstrated that this

process is reversible, because PI3K pathway pharmacological inhibition abolished HOXA gene overexpression in GBM cells. Court et al. [172] also proposed that loss of H3K27me3 at HOX genes is a consequence of genome-wide hypomethylation that characterizes cancer cells. In the mouse, widespread DNA methylation depletion triggers H3K27me3 redistribution [179,180] that in turn leads to dramatic loss of H3K27me3 and ectopic expression at a subset of polycomb target genes, including Hox clusters [180].

Alterations in 3D chromatin structure and HOX deregulation in glioma

Recent studies revealed that alteration in 3D genome organisation is also a feature of glioma cells. Flavahan et al. [178] showed in IDH-mutant glioma that hypermethylation of CTCF binding sites leads to the decrease in the recruitment of CTCF insulator proteins. Therefore, the resulting loss of insulation between TADs leads to aberrant gene activation, such as the oncogene PDGFRA [178]. The generation of 3D genome maps by *in situ* Hi-C in glioblastoma stem cells (GSCs) also stressed that 3D structures are altered in these cells, and also between different GSCs lines, providing a layer of glioblastoma inter-patient heterogeneity (Johnston et al. 2019). Whether this type of alteration also contributes to HOX deregulation in glioma is not documented. However, given the importance of remote regulatory regions and 3D structure in the control of HOX expression during normal development, it is critical to further explore this issue.

In conclusion, HOX clusters display various molecular alterations in glioma cells. To which extent and how each of these alterations contributes to HOX genes gain of expression remain to be determined. Comprehensive studies in which CNVs, DNA methylation, histone modifications and gene expression are analysed in an integrative manner at HOX clusters are now required to decipher the interplay between these molecular signatures in glioma samples and to identify the causes of their alterations. Interestingly, the finding that the HOXA cluster is over-activated in GBM concomitantly with specific HOXB, C and D genes [125,124,122] suggests again that a complex and inter-dependent mechanism of HOX gene regulation might exist in glioma.

4.5. HOX target genes

As HOX proteins are transcription factors and their targets might be the true biological effectors of their functional roles, several authors have tried to identify these targets in GBM. Potential direct or indirect targets of HOXA5, A9, A10, A11, A13, B9, C6, C9, C10, D9, and D10 were already described, and direct targets were confirmed by chromatin immunoprecipitation (ChIP) for HOXA9, A10, B9, C9, and C10. For the sake of comprehensiveness, a brief summary of some potential HOX

targets genes is presented in the following paragraphs (the complete list of potential targets can be found in the original publications).

HOXA5

Cimino et al. [149] identified 692 differentially expressed genes (144 upregulated and 412 downregulated) upon HOXA5 expression in mouse GBM cell lines. By combining this information with data on genes containing HOXA5 binding sequences found by ChIP-seq analysis in human carcinoma cells, they identified 136 common genes (46 upregulated and 90 downregulated). Pathway enrichment analysis of the 46 upregulated genes (e.g. *ASPM*, *AURKA*, *BLM*, *CCNB1*, *CDC25C*, *CDKN3*, *CLSPN*, *FOXM1*, *INCENP*, *MDK*, and *PCNA*) showed that many were implicated in the cell cycle and DNA damage pathways, fitting well with the findings from other studies implicating HOXA5 as a pro-tumour factor.

HOXA9

Microarray analysis of GBM cell lines (U87, U251, and a primary GBM cell line) in which *HOXA9* is overexpressed or silenced, identified more than 3000 deregulated transcripts in U87 and U251 cells, and over 6000 transcripts in the primary GBM cell line [182,150]. Only 61 were altered (18 upregulated and 43 downregulated) in all three cell lines, indicating that gene regulation by HOXA9 in GBM follows a cell-specific pattern. Overexpression of *HOXA9* in normal human astrocytes led to the differential expression of more than 500 transcripts, from which 256 were common to at least one of the GBM cell lines (77 upregulated and 179 downregulated) [182,150]. Pathway and gene set enrichment analyses showed that the HOXA9 differentially expressed genes were enriched, for example, in inflammatory response, cell adhesion/migration, stem cells, DNA repair, and cycling genes. Moreover, *HOXA9*-overexpressing cells showed deregulation of the mismatch repair (MMR) and BCL2 DNA repair systems, as indicated by downregulation of PMS2 and MSH6, and upregulation of BCL2 proteins. Later, Xavier-Magalhães et al. [183] identified *HOTAIR*, a lncRNA located at the HOXC cluster with oncogenic functions in GBM, as a direct target (positive regulation) of HOXA9 by ChIP-PCR. Interestingly, *HOTAIR*, the first described lncRNA to present trans-acting functions, negatively regulates HOXD genes in physiological conditions [63]. Whether this mechanism also occurs or is deregulated in glioma remains to be explored. Recently, Gonçalves et al. [184] demonstrated that WNT6, a ligand and activator of the WNT pathway, which displays oncogenic functions in GBM [185], is transcriptionally regulated by HOXA9, identifying a novel link between HOX and WNT signalling.

HOXA10

Gallo et al. [161] identified, by ChIP-on-chip analysis, 261 direct HOXA10 targets in GBM stem cells, and validated 7 of them by ChIP-quantitative PCR (*HOXA7*, *HOXC10*, *HOXC11*, *HOXC12*, *HOXB13*, *CBX6*, *TERT*, *FGF17*, *JAG2*, and *NODAL*). Enrichment analysis identified significant enrichment for genes involved in processes as growth factor activity, homeobox, CHROMO domain, cytokine, and actin cytoskeleton organization. Additionally, Kim et al. [169] showed that, in GBM cell lines, HOXA10 regulates, in a PI3K-independent manner, PTEN nuclear function (with a different function than the tumour suppressive PTEN cytoplasmic function) through induction of EGR1, which consequently upregulates RAD51 expression, impairing the homologous recombination DNA repair system. In agreement, *HOXA10* silencing increased γ-H2AX, a marker of DNA double strand breaks, in GBM cells [169].

HOXA11

Silencing of HOXA11, which presents tumour suppressive functions in GBM, led to more than two-fold changes in the expression of 62 genes (11 upregulated and 51 downregulated) in GBM cells [147]. This gene list was significantly enriched in genes implicated in the regulation of growth and mitochondrion activity. Specifically, *TGFBR2*, *CRIM1*, *DPYSL2*, and *CRMP1* were downregulated upon HOXA11 silencing, whereas *TXNIP*, *CD22*, *EPCAM*, *MMP3*, and *SLC16A6* were upregulated.

Duan et al. [146] analysed the gene expression profiling data from The Cancer Genome Atlas (TCGA), Rembrandt and the Chinese Glioma Genome Atlas (CGGA) datasets to identify genes showing high correlation (positive or negative) with HOXA13, in order to discover its potential targets in GBM. Positively correlated genes belonged mainly to pathways related to cancer, focal adhesion, WNT pathway, and cell cycle. On the other hand, negatively correlated genes were mostly implicated in the MAPK, ERBB, VEGF, PPAR, and mTOR signalling pathways. *In vitro*, Duan et al. [146] observed that HOXA13 silencing led to decreased levels of nuclear β-catenin and of phosphorylated SMAD2 and SMAD3. Conversely, the level of phosphorylated β-catenin was increased in the cytoplasm, which is indicative of WNT pathway inactivation. Similarly, Yan et al. [154] suggested that the WNT pathway is regulated by HOXC6, through downregulation of WIF-1, a WNT antagonist.

HOXB9

TGFβ1 was identified as a direct target of HOXB9 by ChIP-PCR, leading to increased levels of CD133, OCT4, NESTIN, BMI-1, and, similarly to HOXA13, phosphorylated SMAD2 [153].

HOXC9

HOXC9 has a role in the regulation of autophagy, and DAPK1 was identified as a direct target (negative regulation) by ChIP-quantitative PCR and luciferase reporter assays [165]. HOXC9 silencing released DAPK1 transcriptional repression, resulting in activation of the DAPK1-Beclin1 pathway and autophagy induction in GBM cells. In agreement, the expression of autophagy protein (i.e., ATG7, ATG5, ATG3, and LC3B) was induced by HOXC9 silencing, while no alterations were found in apoptotic-related proteins (e.g., caspase 9/3 and BCL2) [165].

HOXC10

In the context of the growing interest in understanding the complex interactions between cancer and immune cells, a recent study showed HOXC10 plays a role in tumour immune evasion [156]. Indeed, HOXC10 silencing in GBM cell lines decreases the expression of proteins involved in tumour immunosuppression (i.e., TGFB2, PDL2, CCL2, and TDO2). *PDL2* and *TDO2* were identified as direct targets of HOXC10 in these cells by ChIP-quantitative PCR [156]. Moreover, Tan et al. [168] reported a significant enrichment of HOXC10-correlated genes and angiogenic gene sets in GBM patients. Mechanistically, they found that HOXC10 modulates GBM angiogenesis through direct regulation of *VEGFA* (ChIP-quantitative PCR and luciferase reporter assays). Moreover, they showed that PRMT5 and WDR5, which regulate histone post-translational modifications, are required for HOXC10-mediated *VEGFA* upregulation [168]. Thus, although bevacizumab (anti-VEGFA monoclonal antibody) does not significantly improve OS in patients with GBM [186,187], it significantly decreased the growth of HOXC10-overexpressing intracranial tumours in mice and efficiently impaired their angiogenic capacity [168]. This suggests that HOXC10 may be a clinically useful biomarker of bevacizumab response in GBM.

HOXD9

Tabuse et al. [126] performed gene microarray analysis to identify potential HOXD9 target genes in GBM cell lines. *HOXD9* silencing led to upregulation of some genes known to be relevant in cancer (e.g., *TRAIL*, *ANGPTL4*, and *SEMA4D*), and downregulation of others (e.g., *BRCA1*, *TGFB1*, and *BCL2*). Interestingly, BCL2 was also positively associated with another group 9 paralogue, HOXA9, in GBM [150].

HOXD10

Finally, it has been repeatedly reported that, in GBM cell lines, HOXD10 acts by negatively regulating the metastatic-related protein RHOC [173,170,171] and matrix metalloproteinases, such as MMP14 [160,145,171].

Overall, these findings suggest that although the transcriptional target genes of the different HOX proteins mostly do not overlap, they frequently lead to transcriptional programmes that regulate similar molecular processes/pathways (e.g., cell cycle, death, migration/invasion, and inflammation are pathways commonly affected across studies testing different HOX genes), all of which are particularly relevant in the context of cancer. Moreover, it is not surprising that many of the identified targets might be cell type-dependent, as several factors are known to contribute to the diversity and specificity of HOX protein activity, leading to highly dynamic, context-specific HOX transcriptional activation, and to their functional diversification. Indeed, it was described that i) there are many transcriptional partners – HOX cofactors and collaborators – that also present cell type-specific expression patterns [188,189]; ii) the binding between HOX proteins and their cofactors might be context-dependent [190], and different combinations may lead to distinct binding specificities, contributing to the spatiotemporal specificity of HOX proteins; and iii) chromatin accessibility and DNA shape, which may vary between cells, also showed to confer HOX specificity [191]. Bioinformatic studies are needed to cross the thousands of genes and dozens of pathways differentially regulated by each of the 39 HOX proteins in glioma, to better integrate the data. More studies are also needed to identify HOX targets in particular glioma cells of the various tumour niches (e.g., in glioma stem cells versus more differentiated glioma cells; and proliferating versus invading cells), which may be relevant for the design of new therapeutic interventions.

4.6. Clinical implications of HOX genes

In 2008, Murat et al. [124] suggested that a HOX-dominated gene signature, which included *HOXA2*, *A3*, *A5*, *A7*, *A9*, *A10*, *C6*, *D4*, *D8*, and *D10*, was an independent predictive factor of therapy resistance in GBM. In 2010, Costa et al. [122] showed that *HOXA1*, *A2, A3, A4, A5, A7, A9, B7*, and *C6* are overexpressed in GBM compared with non-tumour brain samples, and found that *HOXA9* expression was associated with poor prognosis, independently of other well-known prognostic factors [122]. Indeed, in patients with *MGMT* promoter methylation (associated with better prognosis), *HOXA9* could still identify a subset of patients with poor prognosis [122]. Moreover, the combination of *HOXA9* and *HOXA10* expression was also associated with shorter OS in paediatric patients with GBM [123]. More recently, the prognostic value of *HOXA9* in GBM was consolidated in additional patient cohorts [150]. Although the DNA methylation levels of *HOXA3*, *A9*, and *A10* positively correlate with WHO grading, *HOXA9* and *HOXA10* DNA methylation has been associated with better prognosis in patients with GBM [177]. Conversely, *HOXD8*, *D13*, and *C4* hypermethylation was observed in short-term (<1 year OS) GBM survivors [192]. Additionally, *HOXA5*, *A10*, *A13*, *B9*, *C4*, *C6*, *C9*, *C10*, and *D4* were individually associated with shorter OS in patients with GBM

[149,146,153,157,156,124,165,154,158,162]. Among them, *HOXB9* and *HOXD4* are also associated with shorter OS in patients with glioma, grade II glioma (*HOXD4* only), and grade III glioma [153,158]. In line with their tumour suppressive functions in GBM, *HOXA11* and *HOXB1* were individually associated with longer OS in patients with GBM [159,147].

In the future, these very interesting results should be updated and put into perspective in the light of the new 2016 WHO classification of gliomas that includes molecular features to differentiate glioma subtypes. Moreover, the validation of the prognostic value of HOX genes in several independent, large, and robust datasets, preferably using more robust multivariable statistical analysis, will be of paramount importance to potentially bring HOX genes into the clinics.

Recently, drugs interfering with the PRC2 complex emerged as promising agents for cancer treatment, as the FDA recently-approved drug Tazemetostat for epithelioid sarcoma. While the overall benefit in other tumour types remains to be determined, it is important to consider that PRC2 inhibitors might lead to decreased H3K27me3 at HOX loci, potentially leading to their over-activation. Nonetheless, Khan et al. [193] recently demonstrated that, in opposition to the classical view that PRC2 is the major transcriptional repressive complex of HOX genes, the knockdown of some PRC2 subunits, such as SUZ12, EZH2, or EED, in mouse F9 teratocarcinoma cells, globally decreased H3K27me3 levels, but not in HOX clusters, where curiously H3K27me3 increased instead. Indeed, although decreased enrichment of the PRC2 complexes was observed at HOX loci upon SUZ12, EZH2, or EED silencing, the expression of HOX genes did not increase [193]. MTF2 was pointed as the potential responsible molecule for H3K27me3 enrichment in HOX loci in the absence of PRC2 [193]. Whether a similar effect and underlying mechanism is more generally observed in cancer, including in GBM, remains to be investigated. Additionally, Li et al. [194] described that AC1Q3QWB (AQB), a small-molecule compound, is a selective disruptor of *HOTAIR*-EZH2 interactions, blocking PRC2 recruitment to target genes, presenting a pre-clinical potential worth of further evaluation for the treatment of GBM and breast cancers with high levels of *EZH2* and *HOTAIR*. Indeed, AQB administration to orthotopic mice models of GBM and breast cancer significantly decreased tumour aggressiveness. *In vitro* and *in vivo*, AQB treatment resulted in increased *HOXD10* expression, among other PRC2 target genes. Further experiments in orthotopic mice models of a breast cancer and a GBM patient-derived xenograft (PDX) demonstrated that the combinatorial treatment of AQB with an indirect inhibitor of EZH2 (DZNep) significantly reduced tumour growth and increased mice OS, respectively. However, besides the unknown role of HOXD10 in GBM, and whether it acts as a tumour suppressor or an oncogene, the complete transcriptional profile of HOX genes before and after treatment with these PRC2-targeting compounds remains

unknown. Moreover, drugs like DZNep are now recognized as global histone methylation inhibitors, rather than a H3K27-specific one [195]. These studies emphasize that, in the context of precision medicine, the global identification of the transcriptional signatures of a specific cancer patient should be considered to aim in the decision for particular targeted therapies, as the uninformed use of these agents may lead to pro-tumour effects that will harm a subset of patients.

5. Conclusions and future perspectives

Deregulation of HOX genes is a common phenomenon in malignant primary brain tumours, and most findings reported to date associate HOX gene overexpression with oncogenic functions in glioma. These include critical cancer hallmarks, such as increased cell viability, invasion, migration, angiogenesis, stem cell capacity, and therapy resistance, and decreased cell death/apoptosis (summarized in Fig. 4). Of note, all these studies have not explored the potential role of HOX genes in influencing interactions between cancer cells and those of the tumour microenvironment. Interestingly, Bertolini et al (2019) reported that GBM neurospheres influence non-neoplastic cells from their microenvironment by delivering HOXA7 and HOXA10 via large oncosomes. This large oncosomes, which were abundantly found in the serum of patients with shorter OS, are able to reprogram recipient cells to proliferate, grow as spheres and to migrate. It should also be interesting to investigate how HOX genes may influence tumour immune responses, both in treatment-naïve contexts and under treatment, particularly with immunotherapies. Interestingly, Pojo et al. [150] showed that *HOXA9* downregulates genes involved in antigen processing/presentation in various human GBM [150]. Moreover, Li et al. [156] found that HOXC10 positively regulates genes involved in tumour immunosuppression in GBM, including *PDL2* and *TDO2*. Together with recent reports in ovarian cancer linking HOXA9 to an M2 macrophage tumour-promoting phenotype and reduction of $CD8^+$ lymphocyte infiltration [196], these findings strengthen the idea that HOX genes may play an important role in the regulation of the immune system in the tumour microenvironment.

Considering the critical functional roles and putative prognostic value of specific HOX genes in cancer, including in malignant glioma, and their complex molecular interactions with upstream regulators and downstream targets, it becomes clear that additional studies are necessary to better understand how HOX operate in glioma, and whether they may be therapeutically explored in the clinics. For example, future studies will be critical to identify the whole molecular networks interacting with HOX genes in brain tumours, by generating genome-wide binding profiles to identify their targets, their upstream binding factors (including lncRNA and miRNAs), and protein partners, as well as HOX-specific DNA methylation studies to explore both transcription factor-related and –independent HOX functions. These integrated approaches will broaden our understanding of HOX

roles in normal and abnormal development and malignant transformation, may allow the identification of potentially targetable players suitable for therapeutic intervention, and/or to the development of clinically useful diagnostic/prognostic tools in a paradigm of precision medicine. In addition, it is still unclear whether HOX genes have causative roles in gliomagenesis, or whether their alterations are a bystander consequence of malignant transformation, as well as whether HOX-directed therapies may be useful for the treatment of glioma and other HOX-driven cancers (e.g., acute myeloid leukaemia). These open questions will continue to feed the researchers' interest for many years.

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Figure legends

Fig. 1

HOX expression patterns in the hindbrain and spinal cord

(a) HOX genes are composed by only two exons and one intron. The homeobox sequence, which encodes a 61-amino-acid peptide motif (the homeodomain) with a distinctive helix-loop-helix-turn-helix structure, is within the second exon. **(b)** A colour code indicates the relative area of expression of each HOX gene along the anterior-posterior axis in the developing CNS. The left panel illustrate the rhombomere (r)-specific nested patterns of expression of the Hox1-Hox5 paralogues in the hindbrain. Higher colour intensity indicates higher expression. Positions of motor neuron pools are shown within their rhombomere(s) of origin (Trigem.: Trigeminal; Gl.: Glossopharyngeal). In the spinal cord (right panel), the overlapping Hox4-Hox11 gene expression pattern is shown in relation with the motor neuron columnar organization. MMC: Medial motor column; PMC: Phrenic motor column; LMC: Lateral motor column; PGC: preganglionic motor column; HMC: hypaxial motor column.

Fig. 2

Topological architecture at the HOXD cluster. The HOXD cluster is localized at the boundary of two adjacent topological associated domains (TAD) that each includes an enhancer-enriched gene-desert area. Consequently, the 3' and 5' HOXD genes are controlled by distinct sets of remote enhancers during development. Cen., centromere; Tel., telomere.

Fig. 3

H3K27me3- and H3K4me3-marked domains demarcate silent and expressed HOX genes

Encode-derived ChIP-seq and RNA-seq data show H3K27me3 and H3K4me3 distribution and Hoxa gene expression in mouse A) ES cells, B) embryonic kidney and C) forebrain. In ES cells and forebrain, where all Hoxa genes are repressed, H3K27me3 covered the whole cluster. It may be associated with H3K4me3 at promoter regions, constituting bivalent domains. In embryonic kidney, two H3K4me3- and H3K27me3-marked domains delineate expressed and repressed genes along the cluster.

Fig. 4

Summary of the main functional roles of HOX genes in glioblastoma and their clinical prognostic value.

HOX genes display various critical functional roles in GBM (regulation of cell viability, invasion, migration, apoptosis, cell cycle, colony formation, angiogenesis, resistance to temozolomide and to radiotherapy, and stem cell capacity) that are globally associated with malignancy *in vitro* and *in vivo*. Importantly, some HOX genes have prognostic value in GBM, with putative clinical relevance. Red and blue colours in the heatmap indicate increased and decreased effects, respectively, upon expression of the corresponding HOX gene. Grey colour indicates unknown effects. Empty cells represent absence of the respective paralogue in that cluster. OS, overall survival; TMZ, temozolomide.

Figure 1

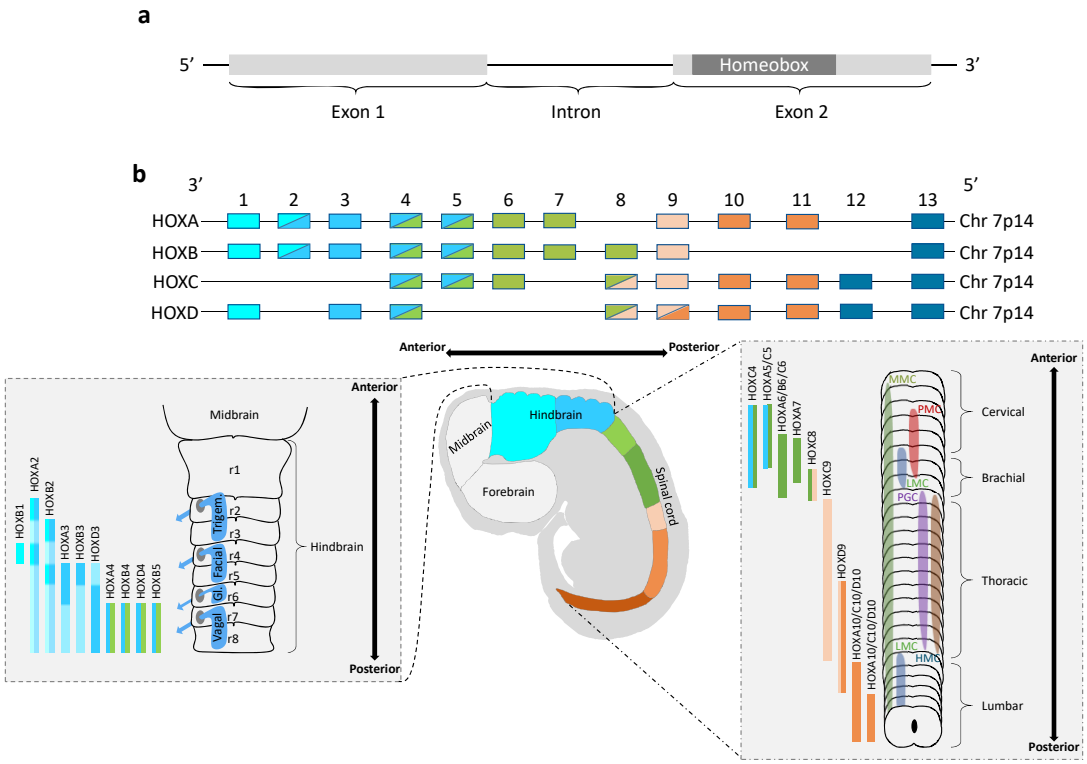

a
5'
Homeobox
3'
Exon 1
Intron
Exon 2
b
3'
5'
1 2 3 4 5 6 7 8 9 10 11 12 13
HOXA
HOXB
HOXC
HOXD
Chr 7p14
Anterior
Posterior
Midbrain
Hindbrain
Forebrain
Spinal cord
r1
r2
r3
r4
r5
r6
r7
r8
Trigem.
Facial
Gl.
Vagal
HOXB1
HOXA2
HOXB2
HOXA3
HOXB3
HOXD3
HOXA4
HOXB4
HOXD4
HOXB5
HOXC4
HOXA5/C5
HOXA6/B6/C6
HOXA7
HOXC8
HOXC9
HOXD9
HOXA10/C10/D10
HOXA10/C10/D10
MMC
PMC
LMC
PGC
LMC
HMC
Cervical
Brachial
Thoracic
Lumbar

Figure 2

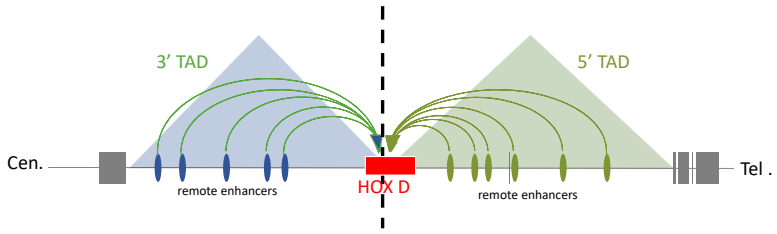


Figure 3

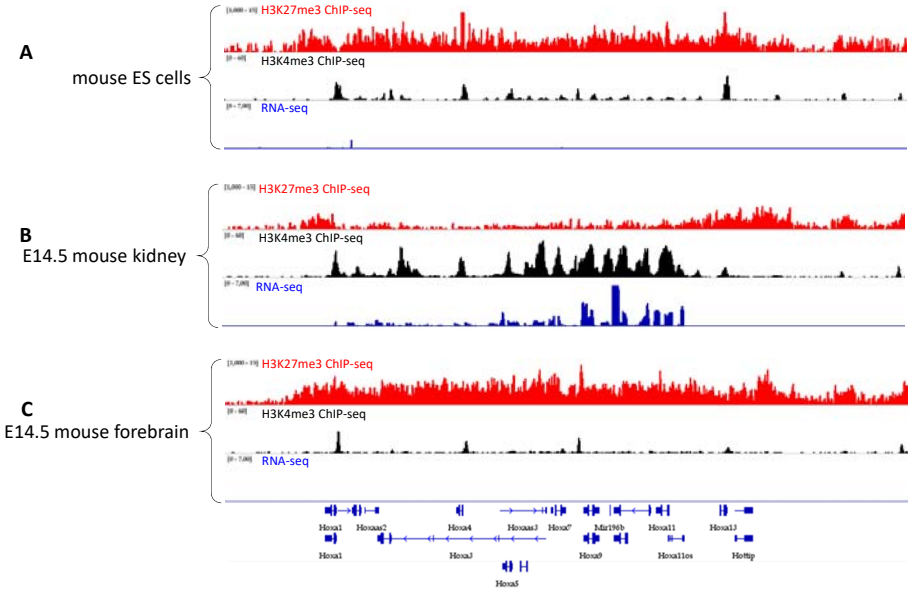
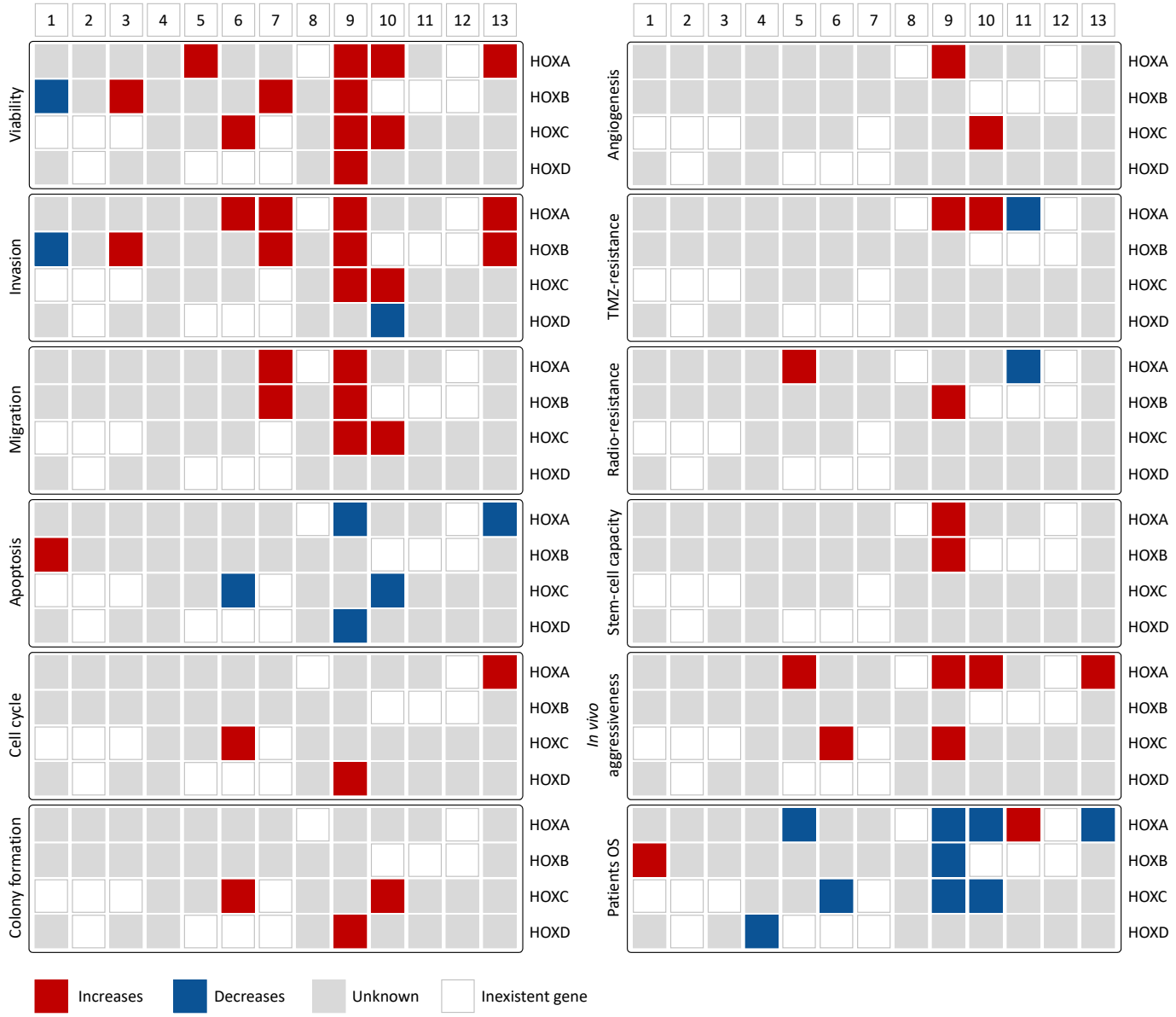

A
mouse ES cells
H3K27me3 ChIP-seq
H3K4me3 ChIP-seq
RNA-seq
B
E14.5 mouse kidney
H3K27me3 ChIP-seq
H3K4me3 ChIP-seq
RNA-seq
C
E14.5 mouse forebrain
H3K27me3 ChIP-seq
H3K4me3 ChIP-seq
RNA-seq

Figure 4


1
2
3
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9
10
11
12
13
Viability
Invasion
Migration
Apoptosis
Cell cycle
Colony formation
Angiogenesis
TMZ-resistance
Radio-resistance
Stem-cell capacity
In vivo aggressiveness
Patients OS
HOXA
HOXB
HOXC
HOXD
Increases
Decreases
Unknown
Inexistent gene