REVIEW ARTICLE

Of travelling homeoproteins and medulloblastomas

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Medulloblastomas are the most common solid paediatric cancers. Their prognosis largely depends on tumour subtype and expression level of transcription factor such as Orthodenticle homeobox 2 (OTX2). OTX2 is an homeoprotein that maintains stemness and initiates oncogenic pathways. Additionally, as many other homeoproteins, OTX2 is able to travel between cells and to modify the transcriptional activity of recipient ones. After identifying travelling proteins in in vivo models, a systematic review of the literature highlighted that at least eleven travelling homeoproteins are associated with medulloblastoma: Cut like homeobox 1 (CUX1), Engrailed homeobox 1 and 2 (EN1 and EN2), Insulin gene enhancer protein ISL-1 (ISL1), LIM homeobox 1 (LHX1), Homeobox protein Nkx-2.2 (NKX2.2), OTX2, Paired box protein Pax-5,6 and 8 (PAX5, PAX6 and PAX8), as well as POU domain, class 5, transcription factor 1 (POU5F1). Overexpression of some of these homeoprotein-coding gene including *OTX2* and *POU5F1* was found to be associated with poor prognosis, while overexpression of *PAX8* seems to have a protective effect, with a significantly better overall and progression-free survival. Research efforts to better understand the transfer mechanisms and intracellular targets of these transcription factors may offer a new range of therapeutics tools, by interfering with these roaming oncoproteins to circumscribe their associated chain reaction of genetic deregulation, or by providing protective homeoprotein supplementation with the aim of stemming tumour development by direct cancer cell penetration and reprograming.

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INTRODUCTION

Medulloblastomas (MB) are the most common paediatric brain malignancies [1]. These WHO grade IV neoplasms of the posterior fossa are classically divided into four main subgroups called sonic hedgehog (SHH)-activated, wingless (WNT)-activated, group 3 and group 4 [2]. These subgroups are associated with different prognosis and are defined by distinct molecular signatures involving transcription factors [3, 4]. Orthodenticle homeobox 2 (OTX2) is notably one of these crucial transcription factors, acting as an oncogene by activation of carcinogenic pathways like mTOR and MYC in MB cells [5, 6]. Interestingly, OTX2 and other similar transcription factors are also homeoproteins, i.e. transcription factors characterised by a conserved 60 amino acid DNA binding domain folded in 3 alpha-helices: the homeodomain [7]. The homeodomain contains two sequences of particular interest named "Sec" (for secretion) and "Pen" (Penetratin, for penetration), since they confer to some of these homeoproteins the ability to be secreted in the extracellular space and to penetrate neighbouring cells in an endocytosis-free manner after an intercellular travel (Fig. 1) [8]. These travelling properties were demonstrated in vitro for more than 150 homeoproteins and in vivo for dozens of them, including OTX2, which is for example able to promote neuroplasticity of the visual cortex or facilitate mitochondrial ATP synthesis after an intercellular travel [9–12].

Unfortunately, the implication of these paracrine mechanisms in brain cancer remains poorly understood. Questioning the potential influence of these oncoproteins roaming inside the tumoral environment, the aim of this review is to identify the travelling homeoproteins associated with MB and recapitulate their known oncogenic and non-cell autonomous roles.

The production of eleven travelling homeoproteins seems dysregulated in medulloblastoma

Based on recent works on travelling proteins in in vivo models, we found 57 human homeoproteins with demonstrated intercellular transfer capacities in mammals [10, 13]. We then systematically reviewed the literature published between January 1981 and September 2024 available on the Pubmed database, by associating each travelling homeoprotein name and the term "medulloblastoma". One hundred and one articles were found eligible for full-text analysis, and we found that at least eleven travelling homeoproteins are associated with medulloblastoma: Cut like homeobox 1 (CUX1), Engrailed homeobox 1 (EN1), Engrailed homeobox 2 (EN2), Insulin gene enhancer protein ISL-1 (ISL1), LIM homeobox 1 (LHX1), Homeobox protein Nkx-2.2 (NKX2.2), OTX2, Paired box protein Pax-5 (PAX5), Paired box protein Pax-6 (PAX6), Paired box protein Pax-8 (PAX8) and POU domain, class 5, transcription factor (POU5F1).

The OTX2 example: a "super-traveller" and a medulloblastoma keystone

OTX2 is known for 25 years to be linked to MB formation. In their 1999 article, Michiels et al. found the gene coding for OTX2 among the transcripts of cerebellum, with a significantly higher expression

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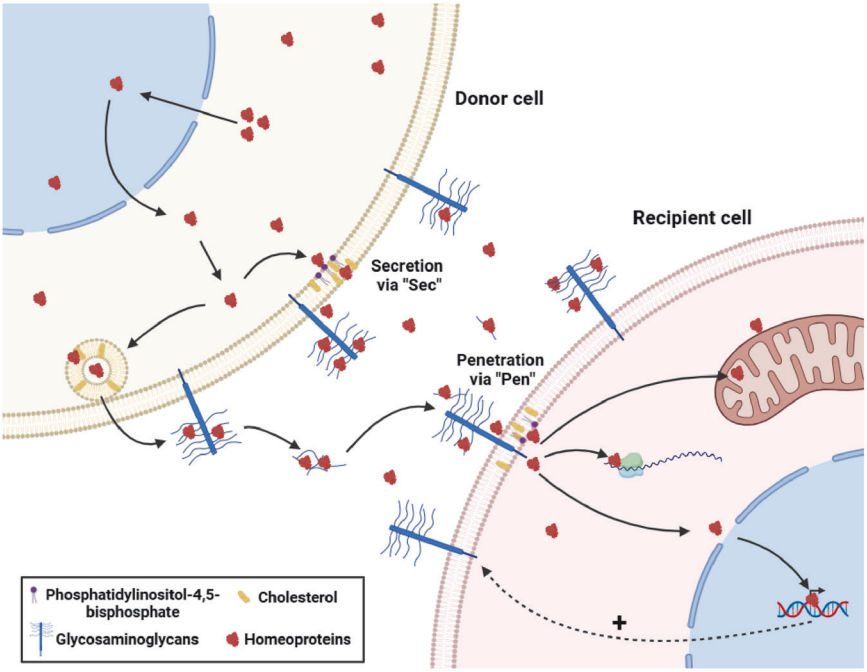

Fig. 1 Schematic representation of homeoproteins intercellular travel. Homeoprotein travel starts by a nuclear import, followed by export into the cytosol. Then, the homeoprotein progresses, partially via cholesterol-enriched vesicles, towards the plasma membrane where it interacts with cholesterol/phosphatidylinositol-4,5-bisphosphate (PIP2)-enriched domains. Once in contact with the negatively charged lipid bilayer, the homeoprotein Sec sequence allows homeoprotein translocation across the cell membrane. When reaching the extracellular space, homeoprotein enters in contact with glycosaminoglycans (GAG), enhancing for example homeoprotein diffusion into the intercellular space via the formation of GAG-homeoprotein hydrophilic complexes. Arriving at the recipient cell, homeoprotein GAG-binding domain recognises and initiates binding with GAG covering the recipient cell. In this configuration of close contact with the phospholipid bilayer, electrostatic interactions between the positively charged residues of the Pen sequence and the phosphate groups promotes insertion of its central tryptophan residue into the lipid bilayer, leading to membrane destabilisation that enables homeoprotein entry inside the recipient cell. Inside the receiving cell, homeoprotein can interact with ribosomes, mitochondria or enter the nucleus, where it can reindorse its role of transcription factor, initiating for example positive feedback loop by increasing GAG production.

in MB compared with the foetal and adult cerebellum [14]. Further studies confirmed this *OTX2* upregulation at both the DNA and RNA levels (due to intratumour dysregulated genetic expression linked for example to genomic aberration with chromosomal amplifications) as well as its role in anaplasia, tumour proliferation and cell cycle alterations [6, 15–17]. Historical series identified *OTX2* overexpression in about 70% of total MB and in more than 90% of MB with anaplastic features, and therefore, poor prognosis [6, 18, 19]. Afterwards, genomic studies identified *OTX2* as an oncogene present in all MB subtypes [6, 20, 21]. Although its role appears to be more crucial in groups 3 and 4 compared to the SHH and WNT-types in terms of tumorigenesis, OTX2 might also have role in tumour maintenance in SHH subgroups [6, 20–22]. High expression of *OTX2* was also found to be associated with the induction and development of leptomeningeal metastases [5, 21].

The oncogenic role of OTX2 is indeed plural, involving several transcriptional mechanisms and disrupting various cellular pathways. First, *OTX2* maintains stemness in its ectopic expression domain leading to failure of a complete cerebellar embryogenesis, via interaction with specific enhancer sequences, regulation of the transcriptional activation of its binding partners (including epigenetic regulators and cell cycle genes), or direct transcriptional repression. OTX2 leads for example to reduction in the production of CBFA2/RUNX1 partner transcriptional co-repressor 2 (CBFA2T2), a part of a protein complex orchestrating proper differentiation of the rhombic lip via recruitment of epigenetic modifiers and transcription factors [3, 23, 24]. OTX2 is also associated with well-demonstrated oncogenic pathways like mTOR or MYC. Indeed, siRNA-mediated knockdown of *mTOR* in group 3 medulloblastoma cell lines significantly reduced OTX2-dependent increase in cell motility, suggesting that upregulated *OTX2* stimulates the mTORC2 signalling pathway by transcriptional activation of the *mTOR* promoter [5]. Moreover, cell treatment with mTOR inhibitors (sirolimus) resulted in a significant induction of cell death in this same model, pointing to a link between OTX2 and group 3 cell survival [5]. Regarding the MYC pathway, it is well demonstrated that *MYC* is commonly amplified in group 3 and 4 subtypes, where OTX2 levels are the highest [25–27]. More than a correlation, there seems to be a direct regulation of the MYC pathway by OTX2. Indeed, in cultured MB cell lines, siRNA experiments to knock down *OTX2* expression led to down-regulated *MYC* expression, while *MYC* knockdown resulted in cell growth inhibition but not to downregulation of *OTX2* [6]. Moreover, direct binding site for OTX2 were identified in the *MYC* promoter [6]. In summary, *OTX2* ectopic expression leads to disrupted rhombic lip differentiation, with maintenance of stemness and activation of key carcinogenic pathways such as mTOR and MYC.

Besides being an oncogene, OTX2 is a transcription factor mandatory for proper embryonic development of the nervous system, and a well-known travelling homeoprotein [12]. This unconventional capacity has been proven in several models, including various type of in vitro and in vivo experiments, as well as rescue protocols with different injection sites (Fig. 2) [10–12]. As an example, Kim and colleagues [12], in experiments using an *OTX2* heterozygous mouse line (*Otx2+/GFP*) to investigate this homeoprotein functions in mammalian visual system, were able to confirm intercellular transfer of OTX2 between different cell types in vivo. Indeed, Otx2 immunostaining signal was present in some T2 OFF-cone bipolar cells that were negative for the GFP reporter and for *Otx2* mRNA. Moreover, via CreERt2 recombination inactivating *Otx2* in specific cells, they could identify the

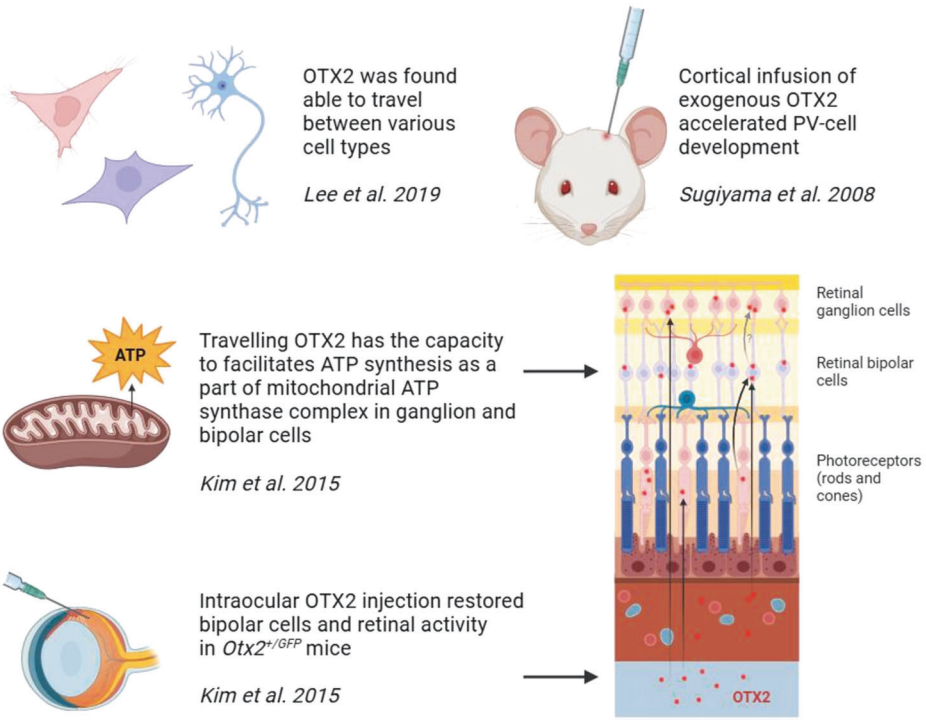

Fig. 2 Known non-cell autonomous roles of OTX2. Schematic representations of the known non-cell autonomous roles of OTX2, including in vitro [10] and in vivo [11, 12] experiment, as well as transcriptional [11] and non-transcriptional [12] post-transfer targets.

photoreceptors as donor cells. More strikingly, they were also able to demonstrate that retinal dystrophy in Otx2+/GFP mice is prevented by intraocular injections of recombinant Otx2 protein, notably by maintaining mitochondrial function (facilitating ATP synthesis) in bipolar and retinal ganglion cells, thereby protecting bipolar cells from glutamate excitotoxicity by avoiding its synaptic accumulation [12].

In the mouse visual cortex, Sugiyama et al. [11]. discovered that intracerebral infusion of Otx2 increases the number of parvalbumin-positive interneurons (PV-cells) (the main class of GABAergic inhibitory neurons of the visual cortex), as well as the number of perineuronal net (PNNs) bearing cells. These findings suggest that Otx2 can modify the transcriptional activity of the recipient cells after an intercellular transfer, and unveil the existence of a potential positive feedback loop between Otx2 and proteoglycans of the PNNs that support homeoprotein travels. This hypothesis of post-transfer modification of transcriptional activity in the visual cortex was subsequently confirmed, with OTX2 directly regulating the Growth Arrest and DNA Damage (Gadd45) gene after cell-to-cell travel [28, 29].

These secretion and internalisation properties of OTX2 may be relevant in MB genesis and progression. Indeed, as described here above, MB formation is strongly associated with high *OTX2* expression, particularly in group 3 and group 4. Taken together, these elements could lead to consider a possible oncoparacrine effect of OTX2, OTX2 being able to leave MB cells and modify the fate of surrounding differentiating cells, maintaining them in a dedifferentiated stage and activating oncogenic pathways (Fig. 3).

ISL1, PAX5 and POU5F1 also act as oncogenes in medulloblastomas

ISL1 is considered as not expressed in the normal adult cerebellum [28]. However, it was detected in 44% of the MB, and its overexpression is known to alter midbrain and cerebellum development [29, 30]. In a series of 25 MB, *ISL1* was expressed in 11 cases (44%): 4 of the 7 desmoplastic MBs (57%) and 7 of 18 (39%) MBs of classical types [30]. ISL1 labelling was nuclear upon immunolabelling, and no relationship with neural differentiation pattern was found [30]. In this same article [30], *ISL1* expression was also significantly correlated with the expression of another homeoprotein coding gene, *PAX5*.

PAX5 is a key transcription factor for neurodevelopment, especially in proper formation of posterior fossa structures like the midbrain/hindbrain boundary, and its knock-out results in disturbed midbrain development [30, 31]. In adult, *PAX5* is still expressed in the neurons of the area postrema of the medulla oblongata and of the periaqueductal grey matter in the midbrain, but is not observed in the adult cerebellum [29, 30]. In a group of 23 MBs, PAX5 was detected in 70% of cases, while not found in the normal cerebellum[29]. Interestingly, in a case of desmoplastic variant of MB, *PAX5* expression visualised using in situ hybridisation or immunochemistry techniques was found to be restricted in the proliferating tumour areas containing undifferentiated cells, and to be absent in the zones undergoing normal neuronal differentiation [30]. However, induced expression of *Pax5* is insufficient by itself to promote development of MB in mice [32].

POU5F1 alias OTC4 (octamer-binding transcription factor 4) is a stem and germ cell marker, and its expression is found in various germinal cancers including seminoma, dysgerminoma, germinoma, and embryonal carcinoma [33]. In MB, *POU5F1* expression was shown to be a predictor of poor outcome [33]. In a group of 37 paediatric patients, median survival time of patients with tumours overexpressing *OCT4* or tumours displaying low *OCT4* expression were 6 and 153 months, respectively [33]. High level of OCT4 was therefore associated with shorter overall survival and predicted high risk for relapse [33].

The ambiguous role of CUX1

CUX1 is an homeoprotein of the CUT family, implicated in embryonic development as well as in DNA damage repair [34]. These two characteristics make of CUX1 a protein of particular interest in embryonic cancers. Furthermore, in the cerebellum, *CUX1* is robustly expressed in proliferating granule cell precursors

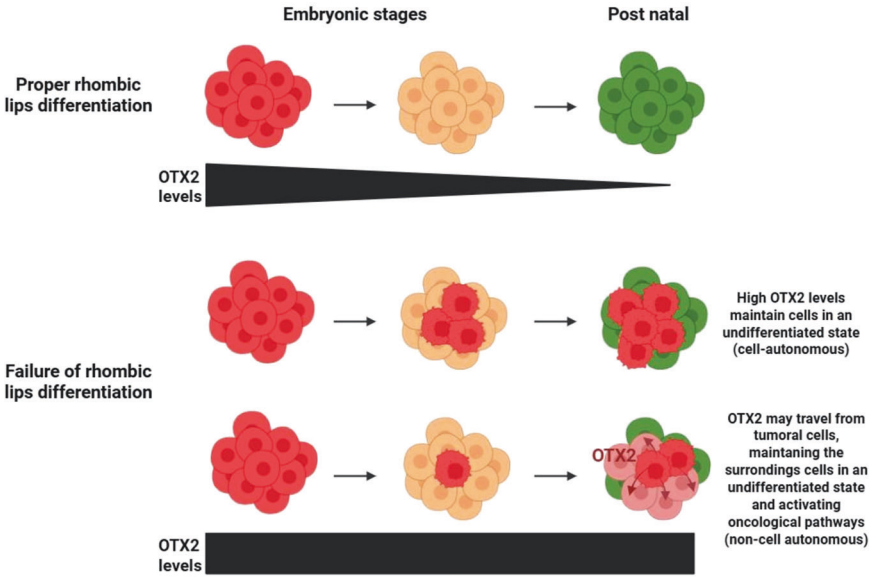

Fig. 3 Schematic representation of OTX2 levels during development and its association with medulloblastoma genesis. Schematic representation of OTX2 levels during development, from high (red), to intermediate (orange) or undetectable levels (green). In medulloblastoma, high-levels of OTX2 as well as stemness are maintained, leading to cellular proliferation. These oncogenic mechanisms may not be exclusively cell-autonomous given the OTX2 travelling capacities, potentially increasing OTX2 levels in non-medulloblastoma cells (pink) and transforming them into cancerous cells.

and in postmitotic, migrating granule cells, while its expression is lost as post migratory granule cells mature. *CUX1* is also overexpressed in a well-established mouse model of MB, as well as in human specimen, but its expression level is lower than in the normal foetal cerebellum [35].

Despite being associated with several cancer, the oncogenic role of CUX1 is ambiguous, since it may act as an oncogene as well as a tumour suppressor [34]. Antisense-mediated reduction of CUX1 levels in MB cell lines led to a decrease in proliferation and altered motility. However, *CUX1* expression correlate with *p53* expression, and high levels of CUX1 are predicted to enhance p53 functionality [35]. *TP53* has been identified as a direct transcriptional target of CUX1, and CUX1 directly interacts with the p53 binding protein, TP53BP1 [35].

Furthermore, Methyl-CpG binding domain protein 1 (MBD1) was identified in a CUX1-related network. MBD1 is found to be associated with DNA repair, and its silencing impairs the activation of the DNA checkpoint response and inhibits DNA repair capacity [36].

Hence, even if overexpressed compared to the normal adult cerebellum, *CUX1* expression in MB is quite low compared to its expression profile in differentiating granule cells. Thus, CUX1 levels in MB may not be sufficient for optimal p53 and DNA damage response, and proper cell cycle control [35].

PAX6, LHX1 and NKX2.2 are linked to the SHH-GLI pathway

PAX6 is a transcription factor that regulates several genes involved in cell fate, proliferation, and neuroectodermal precursor migration during development and remains expressed in normal adult brain [37]. PAX6 was recently identified as a downstream target of the SHH-GLI1 pathway, and SHH regulates *PAX6* expression during early embryonic development (Fig. 4) [37]. In gliomas, PAX6 is considered as a tumour suppressor, inhibiting invasion, and is downregulated by GLI activation. However, in MB cells (Daoy), silencing of GLI1 resulted in a 35% reduction in *PAX6* expression, suggesting that in these cells GLI1 activation stimulates *PAX6* transcription [37]. *PAX6* levels were also investigated in 6 MB cell lines (TE671, PFSK-1, Daoy, TE671c2, D283Med and SK-PNDW) and in 14 primary tumour specimens, with a majority of samples showing high levels of *PAX6* transcripts compared to normal brain tissue [37, 38].

LHX1 is essential for controlling Purkinje cell differentiation in the developing mammalian cerebellum, and its expression is maintained in the adult brain [39, 40]. *LHX1* is globally overexpressed in MB, but its expression significantly varies depending on the subtypes. In fact, *LHX1* expression is generally lower in WNT and group 3 subtypes, but significantly increased in SHH and group 4 subtypes (Fig. 4) [40]. In SHH-activated MB, LHX1 was found to significantly increase the incidence of spinal metastases and of brain leptomeningeal space dissemination [40, 41]. Indeed, in a mouse model with retroviral transfer of SHH and metastasis-inducing oncogenes (including SHH-LHX1 combination), spinal metastases where found in 11% of the mice with SHH expression only, versus 33% for the SHH-LHX1 combination [40]. In addition to increasing the number of metastases, LHX1 also increased their sizes [40]. In the same model, LHX1 was also showed to enhance the local invasiveness of the lesion, for example infiltration of the fourth ventricle [40].

NKX2.2 remains expressed in the normal adult brain, and its expression levels in MB and MB cell lines are generally lower or even undetectable, except for SK-PN-DW cells where *NKX2.2* is upregulated compared to the normal cerebellum [37]. However, alongside *PAX6*, *NKX2.2* was identified as a downstream target of SHH during normal embryonic development as well as in the MB cell line Daoy [37]. Indeed, silencing of GLI1 in these cells resulted in a decrease in *NKX2.2* expression by 50% [37]. These results suggest that in MB with GLI activation, GLI1 may upregulate *NKX2.2* in addition to *LHX1* and *PAX6*. Furthermore, an associated positive feedback loop may also exist since chicken embryonic spinal cord electroporation with a *Nkx2.2* expression vector leads to upregulated expression of SHH in the electroporated cells [42].

The PAX8 counter example: a potential target for rescue therapy?

PAX8 gene expression is associated with forebrain and hindbrain formation, and it is strongly expressed in the neural tube and in the developing CNS [31, 38]. Regarding cerebellum, *PAX8* expression significantly decreases over time in the external granule cell layer but increases in the internal granule cell layer, before being repressed in adults [38, 43]. PAX8 is detected in all the MB subtypes but seems particularly associated with SHH and

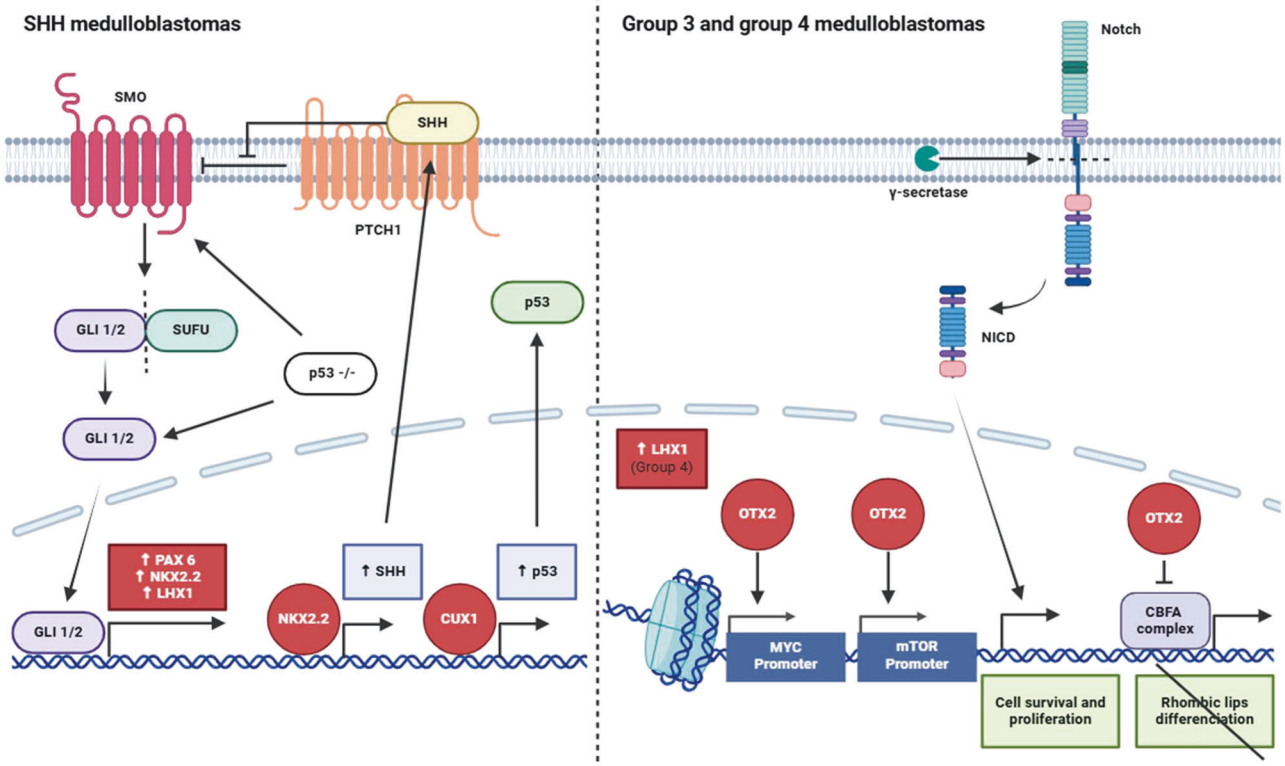

Fig. 4 Interactions between homeoproteins and SHH, group 3 and group 4 medulloblastoma pathways. Left panel : canonical SHH medulloblastoma pathway, where binding of Shh on PTCH1 (Protein patched homologue 1) suppresses its inhibition on SMO (Smoothened), initiating the GLI (glioma-associated oncogene homologue) downstream cascade by dissociating GLI from its cytoplasmic sequestrator SUFU (Suppressor of fused homologue), allowing GLI entry inside the nucleus and increased transcription of *PAX6*, *NKX2.2* and *LHX1* [43]. NKX2.2 increased SHH production, while high level of CUX1 correlated with high level and functionality of tumour suppressor p53 (an inhibitor of the SMO-GLI pathway). $p53^{-/-}$ representing the consequence of a p53 mutation in the SHH pathway, favouring initiation of the SMO-mediated cascade. Right panel: group 3 and 4 medulloblastoma biomolecular findings, where the NOTCH1 pathway is overactivated. A gamma-secretase cleaves the NICD (Notch intra-cellular domain) subpart of the Notch receptor, followed by NICD nuclear translocation and consecutive expression of downstream genes [43]. Additionally, OTX2 is able to bind to *MYC* and *mTOR* promoter, activating the associated pathways, as well as disrupting the CBFA complex. Moreover, *LHX1* was found to be overexpressed in group 4 subtype.

WNT MBs. However, in vitro experiments using Daoy cells demonstrated an increase in adhesion and anchorage-independent growing, as well as a doubling of migration and proliferation rate after *PAX8* siRNA knock-down [31]. Regarding patient outcome, univariate analyses correlated high PAX8 levels with a favourable prognostic factor, associated with a significantly better overall and progression free patient survival [31]. Thus, exogenous increase of PAX8 may be potentially beneficial to reduce medulloblastoma aggressiveness.

Engrailed paralogs are expressed in normal adult cerebellum and in medulloblastomas, and may also have a protective effect

EN1 and *EN2* play multiple roles in cerebellum development and remain expressed in adult life [29, 44]. EN1 is notably important for the transition between neural progenitors and differentiating neurons at the midbrain/hindbrain boundary. Therefore, it is unsurprising that EN factors are detected in a majority of MB [29, 31]. Nevertheless, the expression of *EN1* was found to be quite low across all four subgroups of MBs compared to adult normal cerebellum, and even inferior to physiological levels in group 3 and 4 MBs. Of note, these two transcription factors were never associated with outcome. Yet, EN paralogs may be protective, since loss of En1/2 slightly increases the proportion of undifferentiated granule cell precursors in SmoM2-mutant (mice with constitutive activation of SMO) [44]. Additionally, *EN1* overexpression resulted in *PAX6* downregulation in vivo, consistent with the potential oncogenic role of PAX6 described here above [45].

The non-MB-related effects of these travelling homeoproteins

In addition to their known roles in MB, several of these homeoproteins have, as OTX2, well demonstrated non-cell autonomous functions in the CNS (Fig. 5).

As summarised in the Leboeuf article [13], paracrine EN signalling has multiple important roles in the early development of various organisms, with well-defined donor and recipient cells identified using blocking single-chain antibodies [13, 46]. In drosophila, travelling EN are essential for proper wing formation, inducing anterior cross-vein formation [46]. Roaming EN is also particularly important for the formation of the visual system, regulating retinal ganglion cell axon guidance in the tectum and decussation at the optic chiasma, and shaping the zebrafish optic tectum [47–50]. EN homeoproteins also support cell survival. Extracellular neutralisation of EN1 induced motor neuron death in mice, while exogenous EN restores motor neuron number and activity in *En1*-heterozygous mice and protects mesencephalic dopaminergic neurons from oxidative stress [13, 51].

Regarding the Paired homeoproteins, travelling PAX6 regulates the size of the zebrafish eye anlagen, enhances oligodendrocyte progenitor cell migration in the chick neural tube, and guides Cajal–Retzius cell migration in the mouse neuroepithelium [52–54]. Non-cell autonomous functions of PAX5 and PAX8 remain currently unknown.

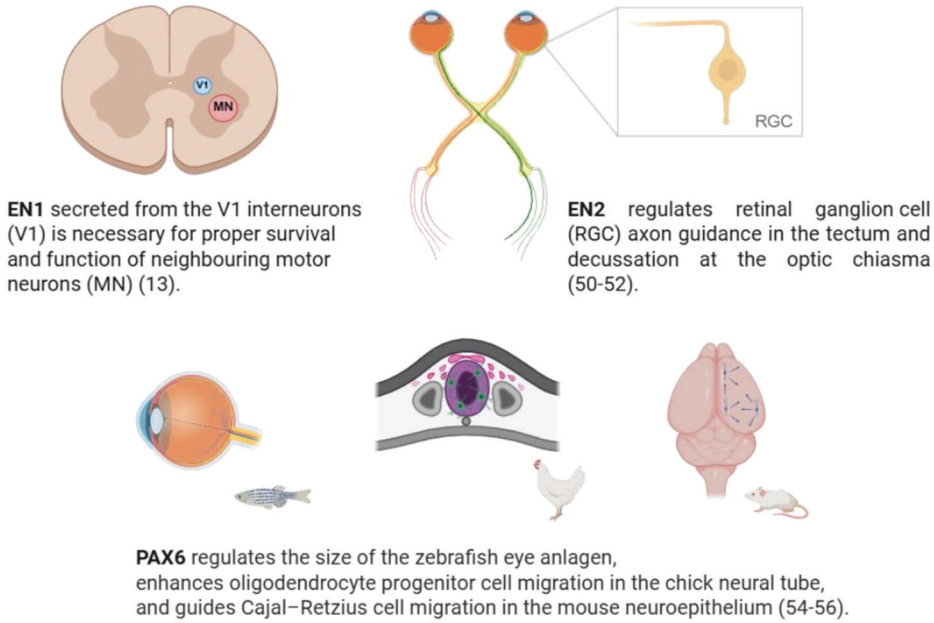

Fig. 5 Different known non-cell autonomous mechanisms of other homeoproteins in vivo. Schematic representation of known non-cell autonomous roles of travelling homeoproteins implicated in medulloblastoma (for OTX2, see Fig. 2).

Non-cell autonomous roles of LIM homeoproteins LHX1 and ISL1 in neural cells have not been described yet. During zebrafish development, ISL1 alongside its paralogs ISL2A and ISL2B may cooperatively contribute to exocrine pancreas extension in a non-cell autonomous manner, but this mechanism was not proven to be a direct consequence of homeoprotein transfer [55].

POU5F1 (OCT4) is one of the most well-known homeoprotein in the context of dedifferentiation. Indeed, it is one of the Yamanaka factors used to reprogramme adult fibroblast into pluripotent stem cells [56]. Moreover, when produced alone in certain type of cells, POU5F1 is able to induce pluripotency by itself [57]. However, despite its ability to travel between cells and in the mammalian brain, non-cell autonomous associated mechanisms remain unknown.

Finally, data about non-cell autonomous effects of CUX1 are rare. It was demonstrated in a mouse model to be involved in the control of cell lineage specification during hair follicle development through both cell-autonomous and non-cell autonomous mechanisms [58]. Nevertheless, as for ISL1, these non-cell autonomous mechanisms may be due to signal regulation, and no Cux1 cell-to-cell transfer was demonstrated in this very model.

DISCUSSION

The unconventional travelling properties of homeoproteins add an additional layer of complexity in numerous diseases, and perhaps more particularly in cancers. Regarding the major cell-autonomous roles of transcription factors in oncological diseases, the fact that some transcription factors can potentially leave their producing cell and travel to the surrounding ones to initiate transcriptional or non-transcriptional functions is a new paradigm in our understanding of neoplastic processes. To date, numerous questions remain open, since various mechanisms associated with these transfers, including some of the most basics ones, remain are still incompletely described or unknown.

First, homeoprotein production or secretion capacities seem to significantly vary depending on the producing cell type. OTX2 was for example barely detectable in the cell culture medium when produced in HEK or MDCK cells, but abundantly found when produced in neural cell type GT1-7, while secretion capacities seemed similar between HEK, MDCK and GT1-7 for all the other homeoproteins implicated in MB [10].

Regarding fluids allowing homeoprotein transfer, CSF, vitreous humour and various cell culture media were proven to be effective carriers. Unfortunately, data about homeoprotein half-life in these different fluids, as well as on blood travel capacities, are missing [10–12, 59].

During their travel, homeoproteins interact with proteoglycans while exiting the donor cell, as well as before penetration in the recipient one (Fig. 1), where some homeoproteins as OTX2 get literally trapped inside the perineuronal nets covering the recipient cells [60]. This affinity for GAG seems mediated by GAG-binding sequences containing doublets or triplets of basic amino acids [13, 60–62]. Two consensus sequences XBBXBX and XBBBXXBX (were B are basic residues and X represent non-basic amino acids) were notably identify in 21 heparin-binding proteins, and are also frequently found in homeoproteins (Fig. 6) [61]. Interestingly, even basic, histidine residues are rare in these GAG-binding domain, as well as in the Pen sequence (Fig. 6) [61]. Surprisingly, we were not able to identify sequences similar to any of these two consensus in PAX5 and PAX8, being either absent or mutated in PAX5 and truncated in PAX8 (Fig. 6). Nevertheless, the XBBB/DKRK (part of the XBBBXXBX sequence) motif found in PAX8 seems to contain the residues that are sufficient for GAG binding [61]. This binding motif as well as interaction with GAG seem indeed important for proper homeoprotein internalisation, since an RK-AA mutation in the GAG-binding sequence of OTX2 (Fig. 6), as well as digestion of the GAG side chains with chondroitinase, lead to significant decrease in OTX2 internalisation inside the PV-cells of the primary visual cortex [60]. Moreover, these GAG binding sequences are also essential for homeoproteins in terms of target selectivity since, in contrast to this decreased OTX2 internalisation observed in PV cells, the same RK-AA mutation leads to increased accumulation of OTX2 inside calretinin-positive interneurons of the visual cortex [63]. These interferences with paracrine OTX2 signalling results in significantly impaired adult visual plasticity [60, 63].

After interaction with GAG-binding domain and a penetratin-mediated internalisation, which is a biphasic process consisting firstly in charge neutralisation between negatively charges of

Homeoproteins	position	Pen sequences																position	GAG sequences							
OTX2	Homeodomain (38-97)	S	R	V	Q	V	W	F	K	N	R	R	A	K	C	R	Q	35	P	R	K	Q	R	R		
CUX1	Homeodomain (1244-1303)	S	T	V	I	N	W	F	H	N	Y	R	S	R	I	R	R	1132	T	K	R	V	K	E		
																		68	S	K	R	S	K	E		
ISL1	Homeodomain (181-240)	R	V	I	R	V	W	F	Q	N	K	R	C	K	D	K	K	231	N	K	R	C	K	D		
LHX1	Homeodomain (180-239)	R	V	I	Q	V	W	F	Q	N	R	R	S	K	E	R	R	178	A	K	R	R	G	P	R	T
NKX2.2	Homeodomain (128-187)	T	Q	V	K	I	W	F	Q	N	H	R	Y	K	M	K	R	183	M	K	R	A	R	A		
PAX6	Homeodomain (210-269)	A	R	I	Q	V	W	F	S	N	R	R	A	K	W	R	R	206	L	K	R	K	L	Q	R	N
																		259	N	R	R	A	K	W		
PAX5	Partial homeodomain (222-254)																									
PAX8	Partial homeodomain (221-253)																	194	D	K	R	K				
POU5F1	Homeodomain (230-289)	D	V	V	R	V	W	F	C	N	R	R	Q	K	G	K	R	280	N	R	R	Q	K	G		
EN1	Homeodomain (303-362)	S	Q	I	K	I	W	F	Q	N	K	R	A	K	I	K	K	291	T	R	K	L	K	K		
EN2	Homeodomain (244-303)	S	Q	I	K	I	W	F	Q	N	K	R	A	K	I	K	K	232	S	R	K	P	K	K		
																		244	D	K	R	P	R	T		
																		294	N	K	R	A	K	I		

Fig. 6 Putative Pen and GAG sequences of travelling homeoproteins present in medulloblastomas. Putative Pen and GAG sequences and their location in the homeoprotein. Homeodomains were aligned according to Holland et al. [65]. Basic residues are highlighted in blue while the cardinal tryptophan of the Pen sequence is showed in orange. OTX2 and EN1 sequences coming from Prochiantz and Di Nardo and Leboeuf articles [13, 62].

phospholipids and basic residues, and secondly in a cell membrane destabilisation by a cardinal tryptophan, cell nuclei seem not to be the only targets of these roaming transcription factors [64]. Homeoproteins like EN or OTX2 where notably found to localise within mitochondria, and capable to interact with members of the electron transport chain and the Na/K-ATPase, but it remains to determine if this affinity for mitochondria is shared by other homeoproteins [12, 13]. Regarding transcription factors reaching nuclei after an intercellular travel, they retain, as described here above, transcriptional activity [11, 13]. This transcriptional activity probably also participates in homeoprotein transfer feedback loops, since for examples direct infusion of Otx2 in the visual cortex leads to increased amount of PNNs around recipient PV-cells [11]. Unfortunately, only sparse data, especially on the other feedback mechanisms taking place, are available, as well as on the association or the compensatory mechanisms between transcription factors (like paralogs) after an intercellular transfer.

We also know that, depending on the cancer type, transcription factors may act either as a protooncogene or as a factor of good prognosis. It remains to elucidate if an intracellular oncogene remains an oncogene after an intercellular transfer, and if the paracrine effects on the recipient cells also vary depending on cancer types. Finally, it remains unclear if, after a first trojan horse manoeuvre, a same travelling homeoproteins is also able to leave the recipient cell and continue its journey, hitch-hiking between successive cells.

Additionally, this article focuses only on homeoproteins with demonstrated travelling properties in vivo. Some homeoproteins that were only demonstrated to roam in vitro and are implicated in rhombic lip development like HOXA5 are highly likeable to also travel in vivo, and may be targets of particular interest. There are also homeoproteins implicated in medulloblastomas formation that were not even tested in vitro for intercellular travel, like PAX2, which is also likely to transfer between cells considering its high similarity with MB implicated homeoproteins and in vivo travellers PAX5 and PAX8 [38].

Regarding treatment strategies, there seem to be two main approaches in homeoprotein-related diseases: transfer abolition by blocking agents in cases of upregulation, or homeoprotein supplementation in cases of defective homeoprotein production.

An obvious criticism on blocking homeoproteins transfer in disease is that it does not prevent the cell-autonomous mechanisms to take place. However, even possibly less important than the cell-autonomous processes, the contribution of these non-cell-autonomous mechanisms may be nevertheless significant, potentially contributing to target cell survival. Indeed, Leboeuf et al. notably demonstrated that EN1 secreted from the V1 interneurons is necessary for proper survival and function of neighbouring motor neurons, extracellular EN1 neutralisation leading to their degeneration [13]. Hence, even if the potential impact of inhibiting homeoprotein transfer in medulloblastoma remains entirely to be investigated, a therapeutic possibility consisting in extracellular sequestration of oncogenic travelling homeoproteins may at least contribute to circumscribe some cancers.

Theoretically, diseases caused by an insufficient homeoprotein production seem the ideals candidates to benefit from theses research efforts. Indeed, regarding their internalisation properties, exogenous homeoproteins should theoretically be able to freely penetrate the targeted cells and restore a possibly deficient non-cell autonomous mechanisms, but also partially rescue cell-autonomous functions in cells where its production is altered. Direct rescue experiment, for example using EN1 injection in a EN1 heterozygous mouse line, showed significant and long lasting improvement of their motor capacities as well as neuroprotection against oxidative stress after intrathecal injections [13, 51]. In the case of MB, we found that PAX8 is detected, but also that high levels of expression are a factor of better prognosis. It remains to elucidate if, in this specific situation, an intrathecal injection of PAX8, artificially rising PAX8 concentration in the tumoral environment, will target cells of interest and will be as effective as EN1 or OTX2 rescuing knockout models [12, 13]. Numerous questions are pending regarding the injection itself. First, should we use the wild-type protein, or should we try to introduce mutations to increase its efficacy (although without disrupting its transcriptional activity), for example by correcting the partial GAG-binding domain to increase its avidity for tumour cells, or by introducing the canonical WF motif of the penetratin sequence that is lacking in PAX8 (Fig. 6). Regarding the injection itself, dose, timing and location remain to be established. In EN1 rescue model, one to two dose of homeoproteins at the concentration of 1 microgram in 5 microliters were intrathecally injected at the L5 level of the spine [13]. In case of MB, intraventricular injection via Ommaya reservoir, direct intraoperative intratumoral injection or via a let-on site catheter may be favoured. In Leboeuf article [13], a

single injection of homeoproteins had significant effects lasting for months, and a second injection performed 3 months after the first one seemed to provide even better results.

At least eleven travelling homeoproteins, including OTX2 and PAX8, are involved in MB formation. These roaming transcription factors mainly act as oncogene in MB, have the ability to activate multiple oncogenic mechanisms, and also to enter and modify the transcriptional and non-transcriptional activities of surrounding cells in several models. Unfortunately, the non-cell autonomous role of homeoproteins in MB, as well as characterisation of their key interactors like GAGs, remain poorly investigated. When established, interference with these transfer mechanisms, via blocking agents like antibodies targeting these transcription factors, could become a promising tool to circumscribe the potential chain reaction of deregulation associated with these travelling homeoproteins. Alternatively, when high homeoprotein cell concentration is associated with favourable outcome, such as PAX8 in MB [31], exogenous homeoprotein supplementation via intrathecal injection may constitute a promising tool, in view of the positive results in neurodegenerative animal's models when rescuing a missing homeoprotein causing the disease [12, 13].

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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