

Review

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Different E-box binding transcription factors, similar neuro-developmental defects: ZEB2 (Mowat-Wilson syndrome) and TCF4 (Pitt-Hopkins syndrome)

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How to cite this article: Meert L, Birkhoff JC, Conidi A, Poot RA, Huylebroeck D. Different E-box binding transcription factors, similar neuro-developmental defects: ZEB2 (Mowat-Wilson syndrome) and TCF4 (Pitt-Hopkins syndrome). *Rare Dis Orphan Drugs J* 2022;1:8. <https://dx.doi.org/10.20517/rdodj.2022.03>

Received: 26 Jan 2021 **First Decision:** 17 Mar 2022 **Revised:** 31 Mar 2022 **Accepted:** 2 Apr 2022 **Published:** 9 Apr 2022

Academic Editor: Daniel Scherman **Copy Editor:** Jia-Xin Zhang **Production Editor:** Jia-Xin Zhang

Abstract

ZEB2 and TCF4 are transcription factors (TFs) whose locations in embryos overlap in many sites and developmental phases, including in the forebrain and its cortical neurons. *De novo* mutations cause the phenotypically overlapping, haploinsufficient Mowat-Wilson (MOWS, in the *ZEB2* gene) and Pitt-Hopkins (PTHS, in *TCF4*) syndromes, which currently cannot be cured. Mutant alleles have been mapped and defects documented (also in brain function) in MOWS and PTHS patients. Appropriately designed mouse models and cells derived from these, as well as cellular models including cultured pluripotent cells, enable investigating the genetic and molecular mechanisms underlying the developmental deficiencies that manifest after birth in the nervous systems and their multiple cell types, as well as those of organs other than the brain, in MOWS and PTHS. Biochemical analyses of cell type-specific transcriptomic changes in these perturbation models as compared to control cells, the identification of the intact-factor dependent and direct target genes, and of partner proteins including chromatin modulators, are revealing complex and multiple modes of action that eventually will explain target gene selectivity for these TFs. Both TFs have also been found to operate in acute and chronic diseases and cell-based repair processes after tissue or organ injury. In addition, the defective function also arises from their aberrant gene expression, which will require a deeper investigation of how the transcription of these TF genes is regulated. Furthermore, these two factors genetically and biochemically interact. This review combines the essentials and recent progress for both TFs for the first time, with a focus on MOWS and PTHS.



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Keywords: E-box, Mowat-Wilson syndrome, neurodevelopment, Pitt-Hopkins syndrome, TCF4, ZEB2

INTRODUCTION

Many causal genes have been identified, and subsequently, *de novo* mutations in patient cohorts mapped in syndromes that manifest before, early, or somewhat later after birth with neurodevelopmental defects such as microcephaly, intellectual disability, seizures, and epilepsy. In many of the monogenic neurodevelopmental syndromes, it is striking that the mutation occurs in a gene encoding a brain development transcription factor (TF) or a subunit of chromatin remodeling complexes (e.g., SOX11^[1]; a number of zinc finger TFs^[2]; NR2F1^[3]; chromodomain DNA-binding helicases CHD3^[4] and CHD8^[5]; and subunits of the ATP-dependent Mi-2/nucleosome remodeling and deacetylase (NuRD) co-repressor complex^[6]; see also the review in Ref.^[7]). Here, we discuss two members of different TF-families that have joined this stage: ZEB2 (originally named SIP1, on human chr2q22.3) of the ZEB family and TCF4 of the basic helix-loop-helix (bHLH) family (originally named E2-2, on human chr18q21.2, and not to be confused with T-cell factor-4, renamed TCF7L2).

Heterozygous mutations in *ZEB2* or *TCF4* cause Mowat-Wilson syndrome (MOWS, OMIM #235730) or Pitt-Hopkins syndrome (PTHS, OMIM #610954), respectively. MOWS and PTHS have strong phenotypic clinical overlap, which warrants interest in co-monitoring the recent progress in *ZEB2* and *TCF4* research. Indeed, one starts to compare in detail their multiple functions and explore their possible mechanistic overlap and genetic interaction. As a result, knowledge is emerging that *ZEB2* and *TCF4* may also be modifier genes of one another, as well as act as modifiers in other neurodevelopmental syndromes and psychiatric disorders (for *ZEB2*, see Ref.^[8]; for *TCF4*, see Ref.^[9]). In addition, analyses of *ZEB2*- or *TCF4*-perturbed models range from neuronal and glial cells to other cell types outside of the nervous systems, i.e., the hematopoietic and immune system, cardiac malformation and malfunction, digestive tract pathology, or even cancer. These features may incite clinical geneticists to re-consider the broad defects of MOWS and PTHS patients in adulthood and to follow up on these patients longitudinally from these new angles as well.

EXPERIMENTAL APPROACHES PUT IN CONTEXT

In addition to the initial mapping of *ZEB2* and *TCF4* expression sites in the developing brain, the studies of upstream regulations and downstream effects of each causal gene/protein in neurons and glial cells, and/or the complex(es) these TFs are incorporated in, have become of highest relevance. Such studies are contributing strongly to the emerging principle of their molecular and phenotypic convergence, which is anticipated with other TFs or co-factors to apply more widely to other neurodevelopmental syndromes or neuropsychiatric disorders^[10].

The ongoing studies identify factor-dependent and direct target genes and/or additional protein partners in brain and neural stem/progenitor cells (NSCs and NPCs) during cell diversity creation in the developing brain. Recently, studies have also started to address brain expansion in evolution (in NSCs and NPCs, e.g., for human CHDs^[11], mouse Sox2 and Chd7^[12]; multiple regulatory proteins^[13]; Tcf4, Olig2, and Npas3^[14]; and FOXP^[15]). They are often complemented with epigenetics and chromatin conformation change studies, which contribute to a better understanding of the precise regulation of their gene expression (see reviews^[16-18]; e.g., studies of TRRAP, a phosphoinositide 3-kinase-related kinase present in histone acetyltransferase (HAT) complexes^[19]). They also include studies of post-translational modifications of the factors, often leading to a better understanding of their activities and/or controlled incorporation in - or removal from - complexes. Such global biochemical insight eventually helps to better delineate diseases or syndromes. This is mainly achieved by demonstrating and explaining the selective transcriptional control of

the factor-bound genes, as well as the impact of mutation or changed dosage of the factor on (each of) the factor functions. This also applies to ZEB2/MOWS and TCF4/PTHS.

Often, the protein-coding sequence of interest is genetically perturbed in mice, using many different conditional knockouts (cKOs), which for the brain is facilitated by the large panel of brain cell-type specific Cre-recombinase strains that are available. However, the increased mapping of missense mutations in particular, and single nucleotide polymorphisms (SNPs) and regulatory elements (REs; e.g., enhancers) of the causal gene(s) in patients with neurodevelopmental syndromes, now allows developing faster, more elegant, and more diverse novel cell-based models rather than entire embryos or animals. Furthermore, such embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and NSCs/NPCs derived from these and grown in cell culture can be submitted to differentiation protocols. For early neural differentiation and subtype neuron specification (and still less for terminal maturation of neurons), these protocols work efficiently in 2D and 3D cultures. These include dorsal or ventral types of forebrain cells in spheroids (including fused spheroids) and cerebral organoids^[20-28]. Moreover, prior to differentiation, these cells are amenable to genetic perturbation (e.g., by CRISPR-Cas). Multi-omics approaches can be applied during the entire process (for CHDs^[29]), even at a single-cell level, to address cell differentiation as well as cell heterogeneity and consider interesting questions about cellular plasticity. Perturbations [KOs, but also RNAi-based knockdown (KD)] also significantly made the research move to less reductionist and, therefore, more systems biology studies. This allowed identifying and modeling the gene/protein regulatory networks (GRNs and PRNs), not only at one or more stages but also during all cell state transitions^[30,31], and again at both cell population and single-cell levels.

Here, we illustrate that ZEB2 and TCF4 are excellent TFs to join this experimental stage-as we propose, preferably together-offering exciting insights in healthy neurodevelopment as well as in the respective syndrome they cause.

MOWAT-WILSON AND PITT-HOPKINS SYNDROMES: HISTORY AND MUTATION SPECTRUM

Mowat-Wilson syndrome

Soon after its discovery as one of the first interacting TFs for Smad proteins (major intracellular effector proteins in transforming growth factor type β /bone morphogenetic protein (TGF β /BMP) family signaling), hence its name Smad-interacting protein-1 (SIP1, renamed ZEB2)^[32], three teams of clinical geneticists independently identified *de novo* mutations in ZEB2 in patients with severe ID, typical facial dimorphism, and Hirschsprung disease (HSCR)^[33-35] as the cause of the well-defined, monogenic, and rare Mowat-Wilson syndrome (MOWS)^[36-38]. Mutant ZEB2 alleles have been determined for about 350 patients who display a great variety of clinical features^[39-44]. Typical in MOWS patients are delays in developmental milestones and motoric development, and anomalies in the eyes and teeth. Other features are typical craniofacial malformation, sensorineural deafness, and HSCR, which originate from defects in the ZEB2-positive (+) cells of the embryonic neural crest cell lineage. Excellent clinical reports have been published on various malformations in the central nervous system (CNS) of MOWS patients over a broad age range, including anomalies of the corpus callosum and/or hippocampus, which can be seen by neuroimaging. These reports have also started to document the longer-term evolution of electro-clinical defects such as focal seizures (by video-electroencephalograph monitoring, video-EEG)^[44-46].

The majority (~93%) of severe MOWS cases are caused by large deletions, nonsense mutations, or short deletions/insertions causing a frameshift leading to a premature stop codon. All of these mapped mutations thus far affect the protein-coding sequences and create haploinsufficiency. Rarer (~5%) are frameshift

mutations and in-frame deletions affecting the C-terminal part of ZEB2 only. This raises the possibility that, depending on the location of such mutation, the C-terminally truncated protein is still produced and not subject to nonsense-mediated mRNA decay^[43,44,47]. Up to date, only few missense ZEB2 mutations (~1.5%) have been detected^[48,49]. These two smaller groups of ZEB2/MOWS-confirmed patients, in general, represent the milder and mildest cases, respectively.

The broad spectrum and relative numbers of severe, milder, and mildest cases make it difficult to firmly conclude on genotype-phenotype correlation. Normally, that conclusion would be based on larger panels of often subtly mutated and, therefore, an aberrant function of one or more of the (hitherto known) affected domains within the ZEB2 protein. For example, two mild MOWS cases have mutations in the NuRD interaction motif (NIM, see below), only causing a loss of interaction of this mutant ZEB2 with the NuRD co-repressor complex, and leaving the rest of ZEB2 intact. However, this has already detectable consequences at the level of ZEB2-dependent and direct target genes in the cellular systems where these mutant proteins were tested^[50,51].

To the list of mapped MOWS mutations that affect the protein-coding exons of ZEB2 (in patients now grouped as Class I), variation/mutation of non-protein-coding sequences of the ZEB2 locus have also begun to be added by the clinical geneticists and mapped exclusively to untranslated exons or (distal) REs (the patients being referred to as Class II). Co-operative enhancers active in NPCs were recently identified^[52] via mapping of chromatin conformational changes in the large genomic region encompassing the human ZEB2 and mouse Zeb2 locus (both on chr2q). These neural enhancers are located about 500 kilobases (kb) away from the transcriptional start site (TSS) of ZEB2/Zeb2, in a ~3.5 megabase (Mb)-long gene desert upstream of the gene^[52]. These add to recently identified, different Zeb2 enhancers in fetal and adult hematopoiesis in mice^[53]. Such findings may make clinical geneticists broaden the current characterization of sequenced MOWS alleles, in particular in mild but clinically convincing MOWS patients, wherein no mutation can be found by exon sequencing of ZEB2. In addition, such new non-protein-coding mutations will inspire establishing new cellular or animal models and documenting deviating ZEB2-dependent transcriptomes and ZEB2-containing GRNs in these.

Pitt-Hopkins syndrome

After the first report^[54] in 1978, it has taken quite some time to delineate the few similar syndromic cases and eventually claim the new syndrome Pitt-Hopkins syndrome (PTHS), based on three defects: (i) brain-related defects (including ID, often coming to attention in the first year of life); (ii) craniofacial malformation; and (iii) episodes of hyperbreathing that start in infancy, as well as breath holding and cyanosis. Several teams confirmed that *de novo* TCF4 haploinsufficiency causes PTHS; thus, not the phenotypically overlapping Angelman (OMIM #105830), Rett (OMIM #312750), alpha-thalassemia/mental retardation syndrome, and X-linked (ATR-X; OMIM #301040) syndromes^[55-57], as well as not MOWS, identified only around the year 2000^[33-35]. The situation is somewhat more complicated because of the existence of Pitt-Hopkins-like syndromes 1 and 2 (PTHLS1/2, OMIM #610042 and #614325) caused by autosomal recessive variants in CNTNAP2 and NRXN1, respectively, with patients who in general have milder delays in motor development.

The original identification of the PTHS locus benefited from large (> 1 Mb) deletions on chr18q in patients, enabling the use of SNP arrays for molecular karyotyping, and was further facilitated by sequencing of many coding exons (and intronic flanking regions) of TCF4, one of the candidate genes in this deletion region. Subsequent selection of extra patients with at least two out of the three defects (see (i)-(iii) above), and exclusion of the other similar syndromes, then confirmed the 20-exon (ex) (with ex1 and ex20 considered

non-protein-coding at that time) and at least 360 kb-long *TCF4* gene as the cause of PTHS. Increased numbers of cases thereafter allowed clinically delineating PTHS, aided by the use of two different clinical tests. These diverged due to the difference in weight given to some of the applied criteria (such as facial features, ID, and gait), and the interpretation of the test score also varied with age^[58,59]. PTHS often includes severe psychomotor delay, seizures and epilepsy, and stereotypical and repetitive movements. The same holds for microcephaly, but minor brain abnormalities are revealed by imaging (dysplasia of cerebellum and corpus callosum, small hippocampus, and hyperintensity of temporal lobe white matter). Additional defects are eye defects (early-onset myopia and strabismus), wider spacing of teeth, anomalies of fingers and toes, chronic constipation, and intestinal malrotation^[58,60]. Thus, many of these defects overlap with those in MOWS^[8,9,44-46,58,59].

The wide phenotypic spectrum and possible clinical inclusion bias, in general, have prompted a number of teams to join forces and apply ID-targeted high-throughput sequencing (HTS) of hundreds of genes in ~900 patients with moderate to profound ID, but without the firm mapping of their syndrome^[61]. Doing so, as part of this study, *TCF4* variants were identified in eight of these patients and could be added to two other ones from a previous ID-HTS study. These ten patients were then evaluated via both aforementioned PTHS clinical tests, and five of them scored convincingly positive. These same studies revealed that the *TCF4* mutation rate is 0.7% in individuals with undiagnosed ID, but also that mutations that cause ID are poorly suggestive of PTHS (and therefore had sometimes been referred to as nonspecific ID). Additional large patient cohorts (~280 patients) assembled from 58 publications with both clinical and sequence data yielded another 25 case reports that were re-investigated^[62].

Altogether, this well-coordinated and vast screening made clinicians aware that in PTHS, more precise diagnostic criteria as well as detailed phenotyping were needed. A survey of hundreds of children with PTHS allowed an estimation of missense (~15% of cases), nonsense (15%), and splice-site (10%) mutations. In addition, small insertions/deletions causing a frameshift (30%), larger deletions, and even translocations involving a part of or the entire *TCF4* (see Ref.^[9], and references therein) have been detected. Most missense *TCF4* mutations, which affect all isoforms, map to ex19 (encoding the bHLH domain, see below), although some are located in ex15 (middle part of AD2) or ex18 (Rep or RD), and a few cases of C-terminally elongated *TCF4* in mild PTHS have been described.

ZEB2 AND TCF4: PROTEINS, AND GENES AND THEIR REGULATION

ZEB2 protein/gene structure

Mouse Zeb2 [1215 amino acids (aa); human ZEB2 (1214 aa; ~140 kDa) was after mouse Zeb1, the second identified member of the small family of vertebrate ZEBs. ZEB1 and ZEB2 are structurally highly similar (for a schematic representation of ZEB2, see [Figure 1A](#)): they have a NIM close to their N-terminus and, more downstream, a homeodomain-like domain (HD, but which does not bind to DNA) and a C-terminal binding protein (CtBP)-interacting domain (CID) with four interaction motifs for (the non-DNA-binding) CtBP1/2 co-repressors. ZEB proteins contain two separated clusters of (between ZEB1 and ZEB2) strongly conserved zinc fingers. The N-terminal (NZF, with four fingers) and C-terminal cluster (CZF, with three fingers) enable ZEB proteins to bind with their two hands (and two zinc fingers in each) to DNA. Binding occurs to two separated CACCT sequences^[63,64], often also found as CACCTG known as E2-boxes, and in few cases also presenting as CACANNTG^[8,52] (CANNTG being an E-box). This could make ZEB proteins putative competitors of bHLH TFs for the same E-type boxes, including for the *TCF4*-containing heterodimers that bind to the CANNTG E-box, with a preference for C or G in the N positions^[65]. In the cell nucleus, ZEB2 (not ZEB1) detectably binds to receptor-activated phospho(p)-Smad proteins that act downstream of liganded TGFβ/Nodal/BMP family receptors. In ZEB2's short (defined as 14 aa-long) Smad-

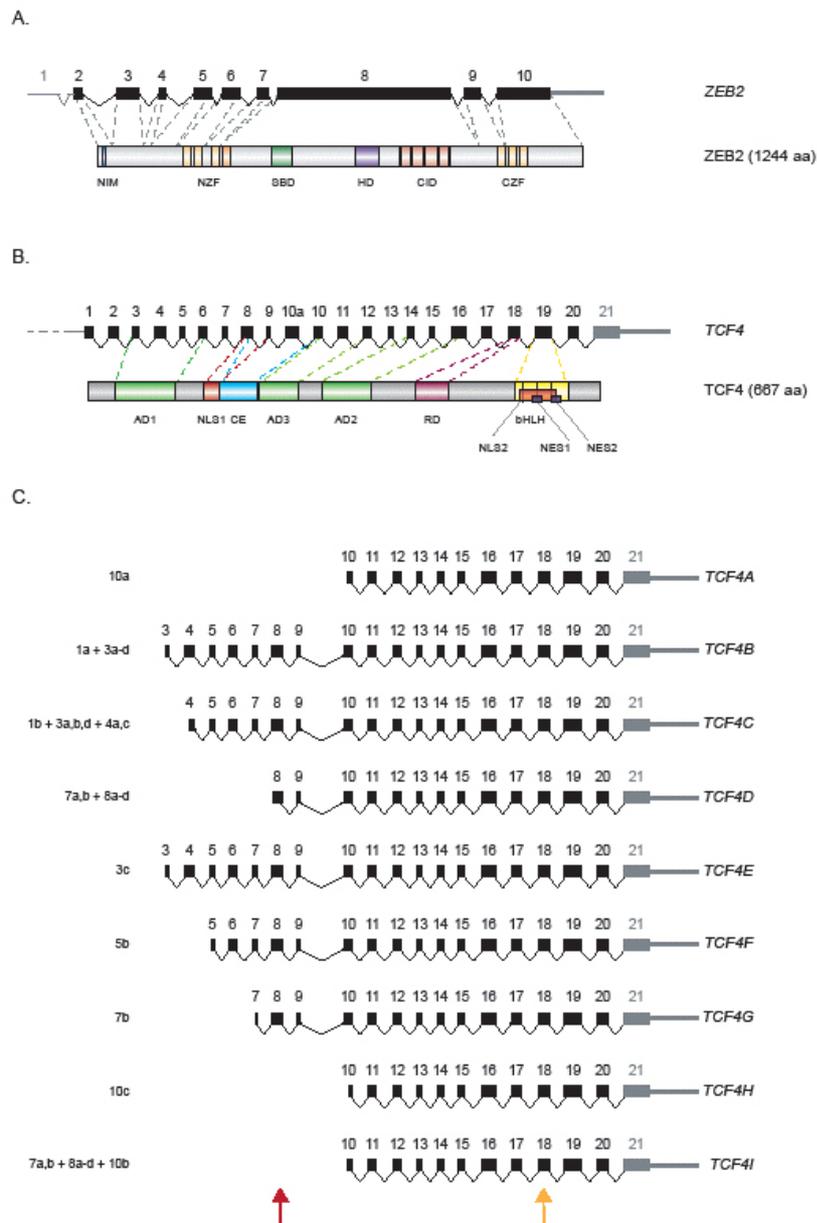


Figure 1. Schematic overview of ZEB2 and TCF4. (A) Representation of (human) ZEB2 gene exons/introns (top) and ZEB2 protein structure (bottom). The largest ex8 encodes for the largest protein segment (in mouse, this corresponds to ex7). NIM: NuRD-interacting motif; NZF and CZF: N- and C-terminal zinc finger clusters; SBD: Smad-binding domain; HD: homeodomain-like domain; CID: CtBP-interacting domain (see Ref.^[8]). (B) TCF4 gene protein-coding exons (20 in total), the thereafter identified 3' untranslated exon (ex21) and introns (top), and TCF4 protein structure (bottom). For reasons of huge complexity, the numerous other identified 5'-non-coding exons and various TSSs (indicated minimally as ...) are not included here. AD: Transcription activation domain; NLS: nuclear localization signal; CE: conserved element; RD: repression domain (sometimes indicated as Rep); bHLH: basic helix-loop-helix DNA-binding domain; NE: nuclear export signal. (C) Alternative TCF4 transcripts as determined for brain. Untranslated regions are not included in this drawing, so only the used protein-coding exons are drawn (in black), together with the one 3'-untranslated ex21 (in grey). On the left, the number/letter combinations indicate that these transcripts also contain brain-specific exons (of which the numbers are given) that are still incorporated in the transcripts, but not coding for protein in these cases, while the letters indicate different mapped splice sites. Names of the transcripts are indicated on the right. The brown arrowhead at the bottom of the panel indicates the region of alternative splicing resulting in a full-length or a so-called Δ variant. The orange arrow at the bottom indicates

the location of the splicing causing the generation of the so-called - and + isoforms. The images in (B, C) are based on the original report^[67] and inspired by reviews^[9,113].

binding domain, a tandem repeat of QxVx is crucial for binding to all p-Smads^[32,66].

In most assays, ZEBs act as transcriptional repressors of tested target genes or transfected promoter-reporter combinations, but, by interacting with histone acetyltransferase (HAT) containing P300 or the related CREB-binding protein (CBP), they can also activate (other) sets of genes (see review Ref.^[52], and references therein). The mouse *Zeb2* locus contains upstream of the first translated exon altogether nine untranslated exons (named U1-U9), which via alternative splicing, are joined to the protein-encoding parts of the *Zeb2* transcript, however, without resulting in *Zeb2* protein variants. The longest protein segment encoding exon in mice is ex7, corresponding to ex8 in humans [Figure 1A].

TCF4 protein/gene structure

The structure of the *TCF4* gene is very complex: through the subsequent mapping of more alternative 5'-exons [20 in total, not shown in Figure 1B], the demonstration of 20 coding exons and the confirmation of one previously unreported 3'-terminal non-protein-coding exon [Figure 1B], the total number of exons in *TCF4* has now risen to 41^[67] (for the equal complexity of mouse *Tcf4* alternative transcripts, see Ref.^[68]; for subsequent reviews, see Refs.^[9,69]; also note that Ensembl lists 48 transcripts) and the size of the human gene accordingly to > 400 kb. Together with alternative TSSs upstream of seven exons in total, this *TCF4* human gene structure is proposed to yield at least 18 protein isoforms (TCF4-A to TCF4-R), which differ at their N-terminus; at least 13 of these are present in the brain. The numbers of different mRNA transcripts are even higher due to alternative splicing within the protein-coding exons [indicated in Figure 1C]. Alternative 5'-exons are used in *TCF4*, also resulting in transcripts that only include ex7-20. To our knowledge, it remains to be established firmly whether human iPSCs or differentiated organoids (or other *ex vivo* models) recapitulate this huge diversity in *TCF4* transcripts, its heterogeneity between cell types, and whether this is also the case in the mouse. This mission announces difficulty, even when using single-cell RNA-seq, for full-length sequencing of transcripts combined with the highest depth of sequencing will be needed.

The first suggestion of the position of the mutation in *TCF4* being relevant to part of the PTHS phenotype, in particular, those aspects being linked to mild non-syndromic ID rather than full PTHS, were mutations in the 5' region of the gene and cassette exons and regions not affecting the important functional domains of TCF4^[70]. Subsequent mapping of *TCF4* mutations in PTHS did suggest genotype-phenotype correlation: those within ex1-4 and ex4-6 would cause mild ID, within ex7-8 severe PTHS albeit without overt facial malformation, and those within ex9-19 typical PTHS^[71]. These have been suggested to correlate with preservation of the intact nuclear localization signal (NLS) and bHLH domains, and with mutation of the NLS and minimally the bHLH domain (encoded by ex18), respectively. Although a genotype-phenotype paradigm has been proposed based on these observations, this has remained rather unclear to molecular biologists. They likely expected extensive documentation of the function of the mutant TCF4 proteins, their heterodimerization with the Class II, cell-type specific bHLH TFs, and their stability vs. degradation. However, significant attempts at addressing their subnuclear localization and dimerization, including in quantitative terms, and activity on target promoters such as *NRXN1β* and *CNTNAP2* promoters have been carried out. These assays indeed pointed out the multiple effects of PTHS missense mutations on TCF4 global function^[72].

HLH heterodimers composed of Class I (seemingly “ubiquitous”, so present in nearly all cell types) and II (clearly cell-type specific) TFs bind to E-boxes in promoters and enhancers (see review Ref.^[73]). Their

affinity to E-boxes is co-determined by co-factors, as well as by Ca²⁺-dependent proteins such as calmodulin that interact with the basic (b) stretch in the (b)HLH domain. In addition, flanking DNA sequences influence the binding affinity of these TFs. Indeed, a subset of TCF4-bound sites has the expanded consensus binding specificity 5'-C(A/G)-CANNTG-3', sometimes with an added downstream outer C(A/G). When present as CpG, both possible additions can, in the mammalian brain, be variably methylated and have positive as well as negative effects on TCF4-binding^[74].

Some consequences and extra complexity

Interestingly, the 5'-C(A/G)-CANNTG-3' motif has also been identified as a binding site for ZEB1 and, due to the high conservation of DNA-binding zinc fingers between ZEBs, been accepted for both ZEB1 and ZEB2^[8,63]. For such motif, the influence of methylation (of a 5'-CG) for binding of ZEBs has not been documented yet. In reverse, it is tantalizing that, via the bHLH domain, TCF4 may be involved in sensing cytosine modification, and that this might also be so for ZEB proteins. Similar to ZEB proteins, TCF4 recruits HATs such as P300^[75-77]. Additionally, in mouse NPCs, Tcf4 binds Mediator and thus co-defines super-enhancers that maintain cell identity^[78].

TCF4 is expressed in many cell types/organs, which classified TCF4 readily as a “ubiquitous” bHLH factor, despite the subsequent identification of cell- and/or tissue-specific presence of the many isoforms [for a schematic representation of TCF4, see [Figure 1B](#)]. Furthermore, the aforementioned complexity of its transcripts means that different TCF4 isoforms [for a selection, see [Figure 1C](#); for recent update, see Ref.^[9]] contain or do not contain domains such as AD1, NLS1, and CE (then referred to as the Δ isoforms), or that the longer isoforms are the only ones that have a complete AD1 [[Figure 1B](#)]. The same holds for alternative incorporation or exclusion of an RSRS-tetrapeptide sequence (named as + are - isoforms, respectively) due to two alternative donor splice sites in ex18. In any case, all *TCF4* transcripts have ex10-20, hence encompass AD3 (ex10-12), AD2 (ex14-16), Rep/RD (ex18), and HLH, NLS2, and NES1/2 (ex19).

All this makes the precise mapping of spatial expression and the estimation of the levels of each of the various *TCF4* transcripts (and Δ and +/- isoforms) very complex and technically very difficult. This is also the case in the brain, but in general, the majority of transcripts are expressed here [these are specifically shown in [Figure 1C](#)], except for those encoding protein isoforms TCF4-J to -N, as these are only present in the testes.

ZEB2 and TCF4 gene regulation

Many studies involving *in vitro*, *ex vivo* (e.g., embryonic brain slices, which can also be subjected to focal electroporation^[79]), and *in vivo* perturbations using single- or two-allele *Zeb2* genetic inactivation (often cKOs) have been carried out (in mouse, about 50 in total). These KO were initially based on the expression domain of *Zeb2* in mouse embryos and early post-natal stages (see review Ref.^[8]). These domains include the forebrain cortex, where *Tcf4* is also expressed in all cells (be it at different intensities depending on the cortex layers, see below), and with *Zeb2* transcripts and Zeb2 protein present in post-mitotic neurons of the upper layers, and both TFs are present in hippocampus. The KO-based studies in cultured cells and mice have also been combined with *Zeb2* cDNA-based (again single- or two-allele) transgene cell-type specific rescue (involving cell-type specific Cre-controlled, from the *Rosa26* locus with inserted *Zeb2* cDNA). This allowed for the creation of various levels of *Zeb2* in the model and obtaining detectable ranges of severity in cellular phenotype. Such experiments have contributed significantly to the conclusion that *Zeb2* dosage, at the transcriptional level, needs precise control. Such control is necessary at every state/stage of *Zeb2*+ cells, for example, during exit from primed pluripotency (in ESCs^[80]), but also in NPCs and other differentiating cell lineages *in vivo*, and/or terminal maturation of these (e.g., natural killer cells^[81] and hematopoietic cell differentiation^[82]).

ZEB2 expression is controlled through promoter-proximal (where TFs such as Smad2, ETS1, HIF1 α , POU/Oct and NF- κ B, E2F1, FoxQ1 and FoxA2, and Fra1/AP1 have been shown to bind) as well as distant regulations located upstream in the ~3.5 Mb-long gene desert (see Ref.^[52], and references therein). In fact, *ZEB2* ranks at the very top of proposed super-enhancer containing genes^[83]. Interestingly, subtle differences in *ZEB2* expression, in spatial-temporal terms and strength, and across species (e.g., human, gorilla, and chimpanzee, with its levels experimentally perturbed in the respective cerebral organoids), influence neuroepithelial transition (hence, also cell shape) and have been proposed to co-determine brain expansion in evolution^[84]. Positive autoregulation of *ZEB2* via a ZEB-binding site in the promoter-proximal region is an important driver of its upregulation during neural differentiation of pluripotent cells *in vitro*^[64]. In addition, an adult-specific remote enhancer 165 kb upstream of the *Zeb2* TSS that binds the bHLH TF E2A/Tcf3 via four E-boxes has been identified and experimentally proven to be critical with regard to the structural integrity of the *Zeb2* locus and its regulation in adult (*vs.* fetal) hematopoiesis. In adults, this enhancer is essential for the production of monocytes, plasmacytoid dendritic cells (pDCs), and splenic B-cells^[53].

Irrespective of the different *TCF4* mRNA variants and protein isoforms and the resulting complexity, a detailed mapping of *TCF4* presence in the brain remains critically needed to start correlating *TCF4* dysfunction with pathophysiology in PTHS. Kim and Berens^[85] constructed and validated a green fluorescent protein (GFP) reporter mouse model that produces a tagged *TCF4*-GFP protein under control of the otherwise unperturbed *TCF4* locus. They specifically achieved this through the insertion of a splice acceptor-*loxP*-P2A-GFP-stop-*loxP* cassette between introns 17 and 18 (upstream of the bHLH domain encoding exons). In doing so, they documented *TCF4* presence in the pallial region and cerebellum of postnatal brain. In the forebrain cortex, *TCF4* is present in both glutamatergic and GABAergic neurons, as well as in astrocytes and mature oligodendrocytes. These observations, highly similar to those with *ZEB2*, strongly suggest that these latter cell types, and not only neurons, should also be studied when assessing *TCF4* pathophysiology. Intronic SNPs in the *TCF4* locus have been identified by GWAS, and one combination (rs9960767 with rs9960767) was found to correlate strongly with schizophrenia (SCZ) and-remarkably-improvement of verbal memory^[86,87]. Jung *et al.*^[88] systematically documented *Tcf4* expression in the developing and adult mouse brain and provided strong support for the role of *Tcf4* in the development and plasticity of cortical and hippocampal neurons. In doing so, they confirmed the structurally similar brain defects between *Tcf4* mutant mice and PTHS patients. They also expanded their *TCF4* localization studies to fetal and/or adult rhesus monkey and human brains and reported highly similar localization between these and mouse brains.

iPSC-derived PTHS patient NPCs and neurons and PTHS patient fibroblasts can also be used as models for studying PTHS^[89]. Such cells also allow investigating how hypomorphic or dominant-negative mutations could cause PTHS. Genetic variation within the *TCF4* locus or intronic SNPs may also cause ID in PTHS or associate with SCZ, respectively. Additionally, more detailed insight in *TCF4* locus control may feed into pharmacological approaches to alter *TCF4* expression in the CNS (and other tissues) and in the cellular models experimentally and document the effects via RNA-sequencing (RNA-seq).

EXPERIMENTAL PERTURBATION AND TRANSCRIPTOMIC PROFILING

Zeb2

Many reviews on *ZEB2*'s dynamic mRNA expression and *ZEB2*'s structure-function and role(s), including in the development of the nervous systems, have been published^[8,90-93]. In short, in mice, *Zeb2* cKOs (*Δex7*, targeted by Cre-recombinase and the critical and largest ex7^[94]) have clearly confirmed *Zeb2*'s role(s) in early CNS development in mammals^[79,95]. Most studies had their focus on the forebrain, but some also

included mid- or hindbrain, or spinal cord, and neural crest-derived peripheral/enteric nervous systems (PNS/ENS; see review Ref.^[8], and references therein). In the forebrain, studies expanded to early post-natal stages^[79,96], adult neurogenesis^[97], and injured brain^[98], as well as in the PNS to (re)myelination^[51,99] and pain sensing (see below).

In more recent studies, adult challenged mice have started to provide important roles of *Zeb2* both as a marker and critical protective or causal gene (e.g., ulcerative colitis^[100], heart infarct^[101], liver steatosis and fibrosis^[102], and melanoma^[103]), thus, often in mouse systems/organs where no embryonic role or homeostatic role has been found, *Zeb2* is not detectable or only at very low level expressed there or then. The recent increasing numbers of phenotypic descriptions of these challenged *Zeb2*-cKO mice, and in their organs or systems, may also urge clinical geneticists to follow up on their MOWS patients in the future in one or more of these extra directions.

• Forebrain development

Good examples of *Zeb2* functions in brain development, with clear consequences for MOWS, came from combining studies of forebrain cortex development and seizures/epilepsy, using a large panel of cortex^[94] or interneuron specific *Zeb2* KOs in the mouse forebrain^[79,96].

Zeb2 deficiency results in insufficient numbers and misguidance of the ventral forebrain GABAergic interneurons caused by increased Dcc receptor and too high Unc5 receptor levels in these cells^[79]. This has been proposed to interfere with long-range Netrin ligand-DCC receptor attractive cues for Unc5, and its levels do provide repulsive signals to Netrin. In the cortex itself, *Zeb2* removal causes aberrant timing of cortical layer formation due to excess and precocious fibroblast growth factor (Fgf9) and neurotrophin (Nt3) production in the *Zeb2*+ upper cortex layers^[95], which are secreted factors that feed back to the *Zeb2*-negative progenitor cells in the developing cortex. This observation, for the first time, identified a cell non-autonomous action mode of *Zeb2*, also in the entire field of brain corticogenesis, where TF-controlled neurogenesis *in vitro* in simple culture media recapitulated the successive waves of neurogenesis observed *in vivo*, leading to the assumption by most workers in the field that the action of the respective TFs was exclusively cell-autonomous.

Furthermore, bulk RNA-seq of *Zeb2*-cKO cells enriched from these respective models has also shown that intact *Zeb2* is needed to precisely control genes involved in neuronal projection and connectivity, synaptogenesis, and synaptic plasticity^[79], but these *Zeb2* functions in these models remain understudied. Reduced levels of excitatory N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid receptors in neurons, however, causes impaired Ca²⁺ signaling upon *Zeb2* deficiency, again indicating a role for *Zeb2* in neurotransmission and synaptic plasticity, possibly underlying late-onset epilepsy^[104]. Further details on these specific phenotypes in these mouse models are again not (yet) available.

Progress may come here from using fused organoids of these cortical neuron types from the respective mouse mutants and eventually (im) balance between neuronal excitation and inhibition as a cause of epilepsy.

• Neural crest-derived sensory neurons

Another interesting example, indicative of the role of *Zeb2* in neural crest-derived cell types, is the reduced thermal pain response observed in adult *Zeb2*^{+/ Δ EX7} mice, whereas mechanical pain was unaffected. This defect is due to reduced spike gain in capsaicin/heat-sensitive dorsal root ganglion (DRG) neurons only,

accompanied by an upregulation of persistent Na⁺ channels and a decrease of delayed rectifier K⁺ channels. The signal propagation from the periphery to the spinal cord was affected as well in these mice. Zeb2 was therefore proposed to regulate thermal pain sensitivity by controlling the transduction properties of nociceptive primary sensory neurons in a novel manner, via changes in DRG voltage-gated ion channels^[105].

The role of Zeb2 in inflammatory and neuropathic pain has been additionally investigated in adult *Zeb2*^{+/ Δ ex7} mice challenged in various ways. Their hypoalgesic phenotype originates from the inflammatory component (hence, not from neuropathic pain) and depends on the Zeb2-controlled development of specific subsets of primary sensory DRG neurons (named C- and A δ fibers)^[105,106]. Therefore, the underreaction to pain observed in some MOWS patients is thought to result from a reduced responsivity to nociceptive stimulation rather than an inability to communicate discomfort. However, this remains, in general, difficult to investigate in these patients.

• Other selected examples

A third selected example is the claimed association of *ZEB2* with SCZ, as different SNPs located in close proximity to *ZEB2* were found in different GWAS studies. One of these SNPs has been proposed to associate with two other psychiatric disorders, i.e., bipolar disorder and major depressive disorder^[107]. These associations might explain some of the emotional disturbances observed in about one-third of MOWS patients and may, according to clinicians, reveal a risk of SCZ in these.

It is important to point out that *Zeb2*^{+/ Δ ex7} mice do not develop histomorphological defects, as well as not at E8.5-9.5 stage, where embryonic lethality in *Zeb2* ^{Δ ex7 Δ /ex7} KOs is seen Ref.^[94]. However, this may depend on genetic background^[108] (see also Ref.^[8]). *Zeb2*^{+/ Δ ex7} adult mice in the appropriate background have craniofacial abnormalities, defects in specific brain regions (corpus callosum and a subclass of neurons in brain cortex), and interestingly (see below, grossly similar to *Tcf4*^{+/-} mice) deviating behavioral activities, i.e., reduced motor activity, increased anxiety, and impaired sociability. Some of these defects are MOWS-like and PTHS-like and are not seen for *Zeb2*^{+/-} mice, other closely related (ICR background) mice, and in outbred genetic backgrounds.

Novel avenues for studying the consequences of perturbed ZEB2 are now possible by deriving iPSCs from reprogrammed somatic cells of MOWS patients with precisely mapped mutation and submitting these cells in culture to neural differentiation over various periods of time (e.g., obtaining NPCs within few days and subsequently the subtypes of neuron as well). Considering many large *ZEB2* deletions that are found in MOWS, the establishment of isogenic control iPSCs by correction of the mutation in patient iPSCs, as often strictly requested by the field, is a major problem. An alternative may be to reproduce such patients' mutations by near-chromosome (as the length of the deletions) engineering in control iPSC lines. Milder cases of MOWS caused by subtle mutations may, because of the aforementioned need for isogenic, endogenous correction, then be investigated first in iPSCs. iPSCs can also be used for establishing longer-term cerebral (forebrain) organoids. Even if (some of) the ZEB2 cell non-autonomous functions would not be perfectly reconstituted in these systems, such iPSCs could still allow transcriptome analysis within *ZEB2* (heterozygous) mutant pluripotent cells, their NPCs, and then derived neurons. The goal here is thus to identify MOWS signatures-or subsignatures, perhaps depending on the type of mutation-that ideally are relevant to neural development and/or could serve patient stratification.

Furthermore, comparing the transcriptome of such MOWS cellular systems with RNA-seq data of differentially expressed genes (DEGs) from the *Zeb2*-cKO mouse models is, in this respect, despite their striking intrinsic differences, already very meaningful. Moreover, such lists of DEGs in mutant cells can also

serve as documentation of (which of these genes are) direct targets of ZEB2, and which of the latter encode for ZEB2 partner proteins, by applying ChIP-sequencing (ChIP-seq, or related strategies) for ZEB2 in combination with ZEB2 interactome studies.

· **Tcf4**

Excellent overviews of the molecular mechanisms of TCF4 in PTHS, sometimes also addressing how these patients are treated and how TCF4 links to other psychiatric diseases, have been published (see^[109], and references therein). Here, we focus on *Tcf4* mutant mice and the consequences for embryonic and adult neurogenesis.

· **Adult neurogenesis**

For adult neurogenesis, a recent observation was made with young adult mice of one of these *Tcf4*^{+/-} models^[110]. Indeed, in these mice, adult hippocampal neurogenesis is severely reduced, presenting as lower stem cell numbers and as impaired proliferation, maturation, and survival of adult-born neurons. Remarkably, these conditions severely ameliorate when the mice are housed long term in an enriched environment, previously known as a powerful stimulant of adult hippocampal neurogenesis (see review Ref.^[111]).

· **Behavior and beyond**

Tcf4^{+/-} (more precisely *Tcf4*^{+/~~b~~HLLH}) mice have been submitted to behavioral testing^[112]. The Δ bHLH (Δ , deletion) approach was chosen because SNPs have been identified that generate a premature stop codon in or a missense mutant of this critical functional domain (i.e., different from other SNPs that associate with SCZ^[113]). Based on observations with PTHS patients, the tests with these *Tcf4*^{+/~~b~~HLLH} mice included motor/balance control and learning, spatial and associative learning and memory, assessment of autism, and extra tests. The Morris water maze, which can be combined with an object location memory task, is a classic hippocampus-dependent task that is used to evaluate spatial learning and memory. In doing so, significant deficits in spatial learning and memory were recorded for the *Tcf4*^{+/~~b~~HLLH} mice. In the same context, hippocampus-dependent fear conditioning tests also revealed a strongly reduced freezing response. To assess autistic traits, the social interaction and repetitive behaviors were documented. These heterozygous mice favor social isolation over interaction, display increased grooming, are hyper-reflexive, and have deficits in prepulse inhibition, which all connect to SCZ and autism due to aberrant sensorimotor gating. The remaining tests revealed preferential use of more left vs. right front paw (when tested on the catwalk) and to put more weight (when tested for dynamic weight bearing) on left front paw. These mice also have weaker grip strength, remarkably in their hindlimbs only, and progressively higher activity in an open field and elevated plus-maze tests.

These already elaborate tests also encompassed RNA-seq on hippocampal CA1 tissue, identifying-based on comparisons that include *Tcf4*^{+/~~b~~HLLH} cells-DEGs related to neuronal plasticity, axon guidance, cell adhesion (not via dysregulated Neurexins), Ca²⁺ signaling, and many upregulated neuroreceptors. The latter indicates that TCF4 (co-)determines the negative regulation of neurotransmitters in the hippocampus. Pathways in learning and memory are also downregulated (e.g., NMDA receptor), as well as those steering myelinations, while reduced global CpG-methylation was found across all gene bodies, but not in promoters. Changes in global demethylation, but here at both TSS and gene bodies, in these mutant mice are significantly linked with specific sets of upregulated genes. Taken together, *Tcf4* haploinsufficiency in these mice clearly affects both expression and DNA-methylation of memory-relevant genes.

· Corticogenesis

It is somewhat surprising that a detailed description of the brain-wide developmental defects in *Tcf4* mutant mice has been lacking for quite some time. The defects include loss of pontine nucleus^[114], promotion of differentiation of adult forebrain NSCs to neurons^[115], and defects in neuronal migration and synapse formation in brain corticogenesis^[116]. In addition, the careful mapping of timing and sites of *Tcf4* mRNA expression, and ideally also the specific *Tcf4* isoforms produced and the estimation of their quantity, is still needed as part of the detailed phenotypes, beyond the admirable efforts already dedicated to this profiling of complex transcripts in the mouse^[71]. In any case, Mesman *et al.*^[117] demonstrated the presence of *Tcf4* mRNAs throughout the mouse brain from E12.5 onwards, but at different intensities: *Tcf4* mRNA levels were found highest in E14.5 cortical plate (CP) of the forming forebrain cortex, and then also at E16.5 in CP and intermediate zone, demonstrating layer-specific *Tcf4* mRNA levels. At the protein level, this layer-specific presence could be confirmed from E16.5 onwards, a time point where most neurons of the developing cortex have been specified. *Tcf4* locates to cells (but not all cells) of layers V and VI, and only very low staining of *Tcf4* is detected in the ventricular and subventricular zone (VZ and SVZ), possibly within cells that have already acquired a neuronal fate.

This same study also impressively combined *Tcf4*^{+/-} and *Tcf4*^{-/-} mice in the analysis of defects in brain cortex architecture. CP thickness is reduced, while at P0, the layers in both mutant mouse models are present, and cell numbers positive for markers for layers V and VI are there (and differ between the two mutants). The cortex layers are also less distinctive, possibly due to aberrant neuronal differentiation and defective segregation of Ctip2+ (corticospinal) and Satb2+ (callosal projection) neurons between E17.5 and P0. There is an indication of the delayed formation of Ctip2+ cells in particular, but also at P0, as based on double-positive Ctip2+/Satb2+ cell numbers. This suggests severe misspecification of the cortical layers, and this has a dramatic impact on the presence of Cux1, which in the mutants is now absent in the upper layers of the *Tcf4*^{-/-} mice but is intact in the VZ/SVZ in all mouse genotypes studied here.

Additional thorough phenotyping also showed that loss of *Tcf4* causes defects in the development of the corpus callosum, the anterior commissure, and hippocampus (at E17.5), which persist until birth. Early phenotyping by RNA-seq of the early E14.5 cortex identified ~130 downregulated genes. These confirm *Tcf4* as a candidate for direct transcriptional activation of genes steering neuronal differentiation and migration and included *NeuroD1*, *Mash1*, and *Id2*. Interestingly, the downregulated genes also encompassed genes related to SCZ or synaptic plasticity. Taken together, many of the defects observed in PTHS patients were recapitulated in the mutant animals, with fairly mild impact in the *Tcf4*^{+/-}, but more dramatic effects in the *Tcf4*^{-/-} mice.

A complicating factor here is possibly that different teams have targeted different critical *Tcf4* exons and/or replaced these with a (non-removed) neomycin resistance cassette. Therefore, other mouse models have proven extremely helpful and informative. Thaxton *et al.*^[118] designed two precise *Tcf4* mutant mice, i.e., a human R580W model (a most common mutation in PTHS, corresponding to mouse R579W) and a model that deletes in-frame three pathogenic R-residues. These mice were compared to ubiquitous as well as CNS-specific *Tcf4*^{+/-} mice. They display common, consistent pathophysiology with regard to microcephaly, deficient spatial learning and anxiety, hyperactivity, and, when comparing some of these mice further, NMDA receptor hyperactivity in the hippocampus. A CNS-cKO (using *hGFAP-Cre;Tcf4*^{fl/fl}) was established^[119] and allowed the study in adult mice of the consequences of complete *Tcf4* removal. This CNS cKO *Tcf4* mouse confirmed the previously documented phenotypes, but also delivered new information, in particular on abnormal neuronal morphology (shortened apical dendrites, increased branching of dendrites).

· Myelinating cells, also in syndromic autism disorders

For both *Tcf4* and *Zeb2*^[84,97], neuronal shape and morphology have clearly emerged as a new topic of interest. As for *Zeb2*, studies of the role of *Tcf4* and how PTHS affects non-neuronal cells in the CNS have been initiated and include oligodendrocyte differentiation and myelination. One study, using five different appropriate PTHS models that show cell-autonomous reductions in oligodendrocyte numbers and myelination, and two extra syndromic autism disorder mouse models (*Pten* and *Mecp2*), showed in an impressive way myelin-related transcriptomic profiles that are shared between PTHS and human autism disorders^[120]. An elegant step to get to this result was to combine the PTHS mouse model DEGs with RNA-seq data from post-mortem autism human brains. As a whole, such studies increasingly confront autism researchers with decisions to embark on CNS myelinogenesis and myelination research. Indeed, the RNA-seq results obtained in the aforementioned multiple *Tcf4* mutants^[120] reveal in the adult brain age-specific DEGs affecting processes in forebrain development, neuron projection, and axon development; post-synaptic density for the upregulated genes in the mutants; and axon ensheathment and myelination for the downregulated genes^[120]. The RNA-seq data thus prompted the validation of myelination deficits in the PTHS mouse models *ex vivo* and *in vitro*, by marker analysis of both oligodendrocyte precursor cells (OPCs) and their differentiated progeny. These experiments were expanded with microscopy to document myelination and with electrophysiology to record the propagation of compound action potentials. They indicated that PTHS mice have reduced myelinated axons, as also seen in specific homozygous *Zeb2*-cKO mice^[51,99,121], resulting in a greater proportion of neuronal activity being transmitted down clearly under- or even unmyelinated axons.

Extra experiments in one of their specific PTHS mouse models (i.e., *Tcf4*^{+tr}, where tr*Tcf4* is proposed to be dominant-negative) clearly indicated that, although the *in vitro* produced OPCs were higher in number than in wild-type *Tcf4*^{+/+} control mice, they still produced fewer oligodendrocytes. Thus, *Tcf4* regulates OPCs, joining *Zeb2* as a critical factor, for promoting OPC differentiation into myelinating cells in the embryonic CNS (and the postnatal PNS). Meanwhile, *Tcf4* function in OPCs has been shown via additional *Tcf4* cKO mice to be critical for medial ganglionic eminence (MGE) and anterior entopeduncular area (AEP)-derived OPCs, the first source of OPCs destined to the olfactory bulb (OB)^[122]. In line with the aforementioned observations, conditional genetic inactivation (using *Nkx2.1*-Cre) of *Tcf4* causes increased numbers of OPCs in the OBs. Furthermore, *Tcf4* suppresses apoptosis by mediating survival in a cell-autonomous fashion (as shown by cell transplantation assays), with apoptosis being controlled by Bax/Bak, Bcl2 family members that are within the nucleus core regulators of the intrinsic pathway of apoptosis, the classical form of programmed cell death, accompanied by mitochondrial herniation. In these mice, it was important to show that the differentiation of (e.g., Lhx1+ and Dlx1+) NPCs remains unaffected in the MGE and AEP upon the loss of *Tcf4*.

· Immune cells

As in the case of *Zeb2*, the role of *Tcf4* is, of course, not limited to neurodevelopment and brain function. *Tcf4* is largely dispensable for T cell and B cell development in the mouse. However, PTHS patients sometimes display impaired interferon (IFN) response and deviating expression profiles in pDCs, a distinct subclass of dendritic cells that rapidly produce type I IFNs in response to virus infection. Elegant transplantation studies using *Tcf4* KO mouse fetal liver cells into lethally irradiated recipient mice, followed by the analysis of the appropriate donor-derived hematopoietic cells, showed efficient reconstitution, giving rise to myeloid, T and B, and DC subsets. However, *Tcf4*-KO derived pDCs were missing from the bone marrow and all lymphoid organs, and splenocytes and the bone marrow cells failed to produce IFN α .

These important results were followed by further studies of the consequences of tamoxifen (TAM)-inducible *Tcf4* KO in mice, with a focus on the analysis of bone marrow and spleens. These studies confirmed the results and also showed that only *Tcf4* has a specific role (unlike two other tested E-family proteins), acts cell-autonomously in pDC development, and displays haploinsufficiency in mice. Thus, *Tcf4* removal in HSCs/HPCs results in failure of pDC development, identifying *Tcf4* as an essential TF in the innate immune system in mice as well as humans. Indeed, in PTHS patients, marker protein deviations and functional defects of pDCs have been documented, boosted by these results in KO mice^[123].

• Cell culture studies

As for ZEB2, other avenues using cultured cells are being used to improve our understanding of TCF4 action in healthy and PTHS brains. Experimental KD of TCF4 by siRNA has been used in both cell lines and NPCs, but initially not always of (fetal) forebrain origin. One study used a conditionally (TAM-induced) proliferative and immortalized (via a c-MycER^{TAM} transgene) cell line from human fetal cortical neuroepithelium, cells of which can differentiate into neurons and astrocytes. They performed successful TCF4 siRNA experiments (using two siRNAs) in these cells that were carefully carried out in the absence of TAM^[124]. Microarray-based transcriptome analysis identified about 1750 and 1500 microarray probes, respectively, that differed as compared to control siRNA, with ~630 of these behaving similarly in the two TCF4 siRNA setups. Candidate genes (39 in total) that survived a stringent analysis, and thus presented as a high-confidence set, were highly enriched for genes involved in cell cycle control.

This same work^[124] also involved the testing for association with SCZ (using large-scale GWAS results from others), leading to five out of nine of those genes to the most significant association (i.e., *GNL3*, *STAG1*, *CENPM*, *NCAPD3*, and *CDC20*), again pointing at cell cycle control. However, while the emphasis of this study is on cell cycle genes, the identified shared DEGs also included genes related to various other functions in the embryonic and adult brain. A second, similar perturbation study, using acute TCF4 KD in the neuroblastoma cell line SH-SY5Y followed by RNA-seq, identified ~1200 DEGs (~500 genes up, ~700 genes down)^[125]. Over-represented genes here are genes involved in the TGFβ-family signaling pathway, epithelial-to-mesenchymal transition (EMT), and apoptosis. Relevant candidates to follow up on are *SNAI2*, *NEUROG2*, and *ASCL1*; the syndrome-causing genes *UBE3A* and *MEF2C*; and, not unexpected, *ZEB2*.

An intermediate approach between *in vivo* and cell culture experimentation, again involving *Tcf4* perturbation, has also been used, i.e., *in utero* electroporation of the cerebral cortex area of E14.5 mice using *Tcf4*-shRNA combined with tracer GFP vectors^[126]. Analysis at P0 identified accumulation of GFP+ cells in the IZ and VZ instead of migrating to the CP, as happens in the controls. Thus, normal *Tcf4* levels are essential for neuronal migration, with likely an underlying aberrant neuronal morphology due to the absence of a leading process, which is already visible at E17.5. The authors excluded that the defect arose from aberrant radial glia organization, division, or survival. Importantly, the *Tcf4* KD in the neurons causes upregulation of *Bmp7*, of which the proximal promoter has multiple E-boxes, with two of these shown to bind *Tcf4*. These observations make *Bmp7* an important effector (that is negatively regulated by *Tcf4*) of neuronal migration. This was further substantiated by additional experiments involving electroporation of expressible *Bmp7*-encoding vectors into control mouse embryos. These results confirm the contribution of *Bmp7* to the phenotype on the one hand and (partial) rescue of the *Tcf4* KD cells obtained by electroporation of *Bmp7*-shRNA on the other hand.

TARGET GENES AND INTERACTOMES

In addition to a meticulous description of phenotypes caused by ZEB2 and TCF4 deficiency in cellular and mouse models and patients, decisive insight into the action mechanisms of these TFs will come from the mapping of their genome-wide binding sites and the identification of co-operating partners or complexes (using proteomics), preferably in neural cells.

Mapping of genome-wide binding sites

No satisfying anti-Zeb2 antibodies are available for efficient ChIP-seq. Recently, mouse ESCs were edited (using CRISPR-Cas) that produce tagged Zeb2-V5 (V5 heterologous epitope) from one of its two endogenous alleles^[64]. ChIP-seq (using anti-V5 antibody) mapped ~2400 Zeb2 binding sites in ESC-derived NPCs, with ~2300 of these annotated to ~2000 protein-encoding genes. Zeb2 is mainly recruited to the promoter-proximal region of transcription regulator or protein modifying enzyme encoding genes, but it also binds to distant REs. The ChIP+ genes include genes classified in Wnt signaling, which is in line with previous observations of DEGs of the Wnt system in *Zeb2*-cKO (CNS) mouse models. A second group included *Dnmt3* family genes, which is in line with previous observations in *Zeb2*-KO ESCs submitted to neural differentiation^[80]. Of the aforementioned 2000 genes, about 1200 are differentially expressed during neural differentiation *in vitro* on Day 4 (epiblast-like stem cells) and Days 6 and 8 (NPCs) as compared to Day 0 (ESCs), including *Tcf4*. Importantly, Zeb2 was also found to potentiate its own expression during NPC production via a promoter-proximal binding site. This autoregulation, as shown by homozygous deletion of the ChIP+ DNA segment (again using CRISPR-Cas), is crucial for proper control of many of its key ChIP+ target genes in ESC-based neural differentiation^[64].

ChIP-seq for tagged V5-Tcf4 in mouse NSCs showed Tcf4 binding to enhancers and super-enhancers^[83], including loci encoding neurogenic bHLH factors, but also-albeit not yielding a strong signal in NSCs-its own promoter^[78]. Tcf4 was found to bind to its targets together with its interaction partners such as Smad4, Sox2, and Chd7^[14]. Gene ontology analysis on Tcf4 target genes, i.e., bound and activated by Tcf4, showed that Tcf4 predominantly controls genes involved in the cell cycle and M-phase of the latter. This could fit with the microcephaly phenotype that is part of PTHS.

Interactomes

Transitions of stem cells into different lineages, and progression of cell differentiation and maturation, including neural cell types, are driven by changes in gene expression regulated by specific TFs that often bind to evolutionary conserved DNA motifs in TSS-proximal sequences (e.g., promoters and first intron) and to distant REs. Transcriptome, epigenome, and higher-order chromatin do not stand by themselves but are intertwined and influence each other herein. This, in collaboration with the actions of chromatin-modulating complexes, results in the proper activation or repression of target genes. Chromatin modulation involves re-modelers and modifying enzymes including methyltransferases, HATs and kinases (often collectively called chromatin writers), demethylases and histone deacetylases (HDACs, erasers), and complexes such as the switch/sucrose nonfermenting (SWI/SNF) complex [also known as BRG1/BRM-associated factor (BAF complex); named readers]. Enhancers are often located *in-cis* kb to sometimes Mb away from the specific genes they regulate, requiring TF- and Mediator (complex)-assisted DNA-looping. Thus, correct gene expression also requires epigenetic reorganization via chromatin modifications and chromatin remodeling.

Already from the earliest studies with Zeb1, ZEBs have been proposed as putative competitors in DNA-binding for E-box-binding transcriptional activators (bHLH TFs) simply by target-site occupancy, soon followed by the first reports of ZEBs being active transcriptional repressors in most assays. For this latter

activity, co-repressor binding has proven important, e.g., with NuRD. It is composed of, among others, at least the ATPases CHD3/4/5, HDAC1/2, metastasis-associated protein (MTA)-1/2/3, retinoblastoma binding protein and histone chaperone RbBP-4/7 (also named RbAp48/46), methyl-CpG-binding domain protein (MBD)-2/3, and zinc-finger TFs GATA2A/2B^[127,128]. For repression, this complex thus combines deacetylation of histone-H3 by HDAC1/2 with demethylation of H3K4 (see review Ref.^[129]). NuRD-mediated chromatin remodeling in cerebral cortex development, neurodevelopmental disorders, and myelination was recently reviewed^[6,29,130,131].

Various subunits of the NuRD complex, including CHD4, were identified in affinity-purified Zeb2 complexes isolated from Zeb2-overproducing heterologous cells^[50]. ZEB2 associates with NuRD via its NIM (see above), but the subunit/s of NuRD that mediates this direct interaction is/are not known. In two aforementioned patients with mild MOWS caused by mutations affecting the ZEB2 NIM, one being a subtle R22G missense mutant, the only direct molecular detectable consequence is that ZEB2 interaction with the NuRD complex is lost. Such aberrant ZEB2 is indeed unable to recruit NuRD (subunits) but, as a result, displays reduced transcriptional repression activity on, e.g., the *XBMP4* gene promoter (in *Xenopus* (*X*) embryos), a target of *XZeb2*. By using a dominant-negative variant of the ATP-binding NuRD component CHD4/Mi-2 (its *in vitro* made sense RNA was injected in 1-4 cell stage *Xenopus* embryos), it was shown that this subunit and thus NuRD is actively involved in repression of the ZEB2 target gene *Cdh1* (encoding E-cadherin, cadherin-1) and in *XZeb2*-controlled neural induction^[50]. This work also showed that the absence of NuRD binding to human ZEB2 can be the cause of mild forms of MOWS. Similar functional consequences (and inability to rescue *Zeb2*-KO) of NIM-mutant Zeb2 were documented in myelination and adult neurogenesis^[51,97]. Cellular systems that enable documenting GWBS for a NIM-defective human ZEB2 are not available yet; hence, target genes that may co-depend or depend exclusively on NuRD-ZEB2 interaction remain unknown.

Tcf4 interacts with more than 100 partners^[14], including chromatin remodeling complexes such as SWI-SNF, NuRD, and TRRAP, but also many TFs. Known partners, including HLH factors Ascl1 (also named Mash1), Id1, and Id3/4, were also identified in this study. Other bHLH factors (Tcf12 and E2-A/Tcf3) bind to Tcf4 as well. Interestingly, Zeb2 was detected in tagged Tcf4, Sox2, and Npas3 interactomes^[14]. A major functional finding here was that Tcf4 binds and activates multiple genes involved in primary microcephaly, such as *Mcph1* and *Wdr62*. It co-localizes on primary microcephaly genes with its interaction partners Smad4, Sox2, Chd7, and Ep300. Smad4 also (co-)activates transcription of *Mcph1* and *Wdr62*. Mutations in *SMAD4*, *EP300*, *CHD7*, and *SOX2* cause microcephaly in at least some patients. Therefore, a picture has emerged that TCF4 is part of a protein interaction network regulating human primary microcephaly genes, in which network members cause microcephaly as part of the human phenotype they cause.

Mediator was not picked up in Tcf4 co-purifications^[14], but *vice versa* Tcf4 did show up prominently in purification of the Mediator complex from NSCs^[78]. Identification of enhancers and super-enhancers in NSCs by Mediator ChIP-seq showed that they nearly completely overlap with genome-wide binding of Tcf4. Moreover, Tcf4 KD lowered Mediator binding to common binding sites, showing that Tcf4 exerts genome-recruitment activity on Mediator^[78]. This co-localization of TCF4 and Mediator is striking, and in fact, goes beyond enhancers, making both Tcf4 and Mediator mark so-called broad promoters^[132,133]. Broad promoter containing genes are strongly linked to cell identity. This suggests that the identified Tcf4-Mediator axis plays a major role in NSCs^[78].

Impact on or by Wnt signaling

The most affected deregulated genes resulting from *Zeb2* deficiency, certainly in the development of the nervous systems, but also in cell culture, often map to the Wnt signaling system. This is the case in mouse hippocampal development^[134], embryonic CNS myelinogenesis, and post-natal Schwann cell (SC)-mediated (re)myelination in the PNS^[51,99,121]. Myelination (see also above) is absolutely required for optimal conduction velocity of nerve impulses, and defects may cause disrupted motor function. It occurs by the formation of myelin sheets generated by oligodendrocytes/SCs, which derive from OPCs. The differentiation of these OPCs toward mature, myelinating cells is inhibited by BMP, Wnt, and Notch signaling^[135]. *Zeb2* protein is present in cells that accompany motor neuron axons in the trunk of early mouse embryos at the level of the limbs^[136]. *Zeb2* was proposed as a strong direct target for the upstream TF Olig2, and *Zeb2* mRNA requires normal levels of Olig1. Olig1 and Olig2 (including their overproduction) also impact neurodevelopmental disorders, even in Down Syndrome and autism spectrum disorders^[137]. Furthermore, Olig1 and Olig2 bHLH TFs are essential for CNS myelinogenesis^[137]. Analysis of *Olig1* and *Olig2* KO mice revealed that in P14 and E14.5 spinal cords, respectively, *Zeb2* mRNA levels become extremely low to undetectable. In cultures of adult rodent early-OPCs, overproduction of Olig1 and/or Olig2 activates *Zeb2*. High levels of *Zeb2* protein are present in mature oligodendrocytes, and only very low levels are detected in OPCs. These observations provided a strong basis for generating *Olig1*-Cre;*Zeb2* cKOs and documenting the effects of *Zeb2*'s removal on CNS myelinogenesis. Such mice are viable, but two weeks after birth, they develop generalized tremors, limb paralysis, and seizures, similar to the symptoms observed in other genetic mouse models with defective myelinogenesis.

Intact *Zeb2* was further shown to be dispensable in platelet-derived growth factor receptor-alpha (PDGFR α)⁺ OPCs, but its presence is critical for their differentiation. Subsequent experiments demonstrated that *Zeb2* downstream of Olig1 and Olig2 is needed during CNS myelinogenesis for differentiation of OPCs to myelinating cells, and that this requires both *Zeb2*'s repressor and activator activities^[8]. Importantly, *Zeb2* represses sets of BMP-Smad-activated genes that normally inhibit myelinogenesis, and *Zeb2* directly activates *Smad7*, which (co-)downregulates BMP signaling with *Zeb2* (via BMP-pSmad interaction). In a Smurf-dependent manner, *Smad7* also sends Wnt-activated β -catenin (β cat), with the latter normally inhibiting myelinogenesis also, into degradation^[121]. Thus, in a cell-intrinsic manner, *Zeb2* is needed to generate anti-BMP-Smad activity and anti-Wnt- β cat effects in CNS myelinogenesis.

Based on this, a number of predictions could be tested and confirmed. One is that, in neonatal OPCs, whose proliferation is stimulated by PDGF-AA, *Zeb2* overproduction would enhance their differentiation to mature oligodendrocytes, as detected by downregulation of genes encoding negative regulators of differentiation, and a strong increase of *Sox10* and *Olig2* expression. Furthermore, other experiments demonstrated that *Zeb2*-P300 co-operation is responsible for direct activation of the pro-myelination genes. Another prediction was that *Smad7* overproduction would rescue the myelination defect in *Zeb2* KO OPCs, which was the case^[121]. These results are the start of new investigations aiming at addressing the *Zeb2* function in SCs of the adult PNS, where *Zeb2* is present^[51]. Sciatic nerve injury already rapidly upregulates *Zeb2* (in a few hours), and *Zeb2* levels remain high in remyelinating SCs for a long time after the injury^[99].

Taken together, these *Zeb2* studies of the cellular and molecular phenotypes in CNS myelinogenesis and PNS myelination are comparable in many respects. They show that intact *Zeb2* is needed to control signaling pathways that impact myelinating cells, i.e., *Zeb2* is needed to generate and maintain anti-Notch/Hey2 and anti-Sox2, as well as (in both CNS and PNS) anti-BMP and anti-Wnt activities in these cells. Rescue experiments with the NIM-deficient *Zeb2*^{R22G} mutant (see above) highlighted the importance of NuRD-interaction. Importantly, these results in the mouse models recapitulate the phenotypes observed in MOWS patients presenting a delay in myelination and defects in white matter, proposed to cause motor

deficiencies such as a delay in walking.

Modulation of Wnt/ β cat active signaling and epigenetic status also caught attention by the TCF4 field^[138]. In the case of signaling, the approaches used Wnt pathway activation via the addition of Wnt3a-containing conditioned media or CHIR-99021, a potent inhibitor targeting GSK3, wherein GSK3 normally negatively regulates canonical Wnt signaling. Both treatments resulted in 1.4-6-fold upregulation of *TCF4* depending on the specific transcripts analyzed, but these are experimentally difficult to allocate via Western blotting, due to the different TCF4 protein isoforms (see above). Further analysis, also involving the annotation of potential TCF/LEF binding sites in TSS-proximal sequences of Wnt-responsive genes, revealed that the highest Wnt-responsive *TCF4* transcripts [encoding a short form of TCF4, TCF4-A; see [Figure 1C](#)] contained multiple TCF binding sites, which could-albeit indirectly-be confirmed for binding (i.e., by ChIP-seq of TCF7L2).

A next approach by the same authors used two different pharmacological inhibitors of Wnt canonical signaling in NPCs stimulated with Wnt-containing conditioned medium (CM) (i.e., the tankyrase inhibitor XAV-939, causing enhanced degradation of β cat, and ICG-001, preventing β cat from binding to the HAT CBP, a member of the P300 family). These treatments were found to downregulate *TCF4* mRNA expression. They also paved the way to test selective HDAC inhibitors, investigate, in particular, the exclusive synergism of Class I HDAC inhibitors in Wnt-CM conditions only, and document these experiments by RNA-seq and TCF4 binding site analyses, in particular for DEGs^[138].

Many of these DEGs are known as critical to nervous system development and function and act in synaptogenesis, post-synaptic density scaffolding and signaling, and axon growth and guidance. In addition, many of these DEGs and other ones have been associated with autism, ID, and/or psychiatric disorders. They represent interesting downstream markers and are part of signatures that can be compared between PTHS patient-derived cells and neurons. Such work is also inspiring for *ZEB2* research into pharmacological modulation that targets *ZEB2* expression regulation from the single remaining intact *ZEB2* allele in MOWS.

ZEB2 and TCF4, each other's modifier for neural differentiation in vitro, but also of other genes in normal brain development?

As illustrated above for *ZEB2* and *TCF4*, but also applicable to other neurodevelopmental TFs, the combination of genetic analysis and mutation mapping, the phenotypic and transcriptomic analyses of appropriate cellular and animal models, and the mapping of genomic TF binding-sites and TF interactomes give insight into TF actions (also in concert with chromatin remodeling complexes) at the level of individual disease genes, as well as GRNs and PRNs, including these steering neural differentiation.

The first example of new insight is that a number of *Zeb2* ChIP+ target genes, at least as mapped thus far in mouse ESC-derived NPCs, could be associated with human neurodevelopmental disorders (such as ID, mental disorders, eye defects, seizures, and speech impairment). For instance, a strong *Zeb2* binding site has been mapped to the PTHS *Tcf4* gene proximal region^[64]. In addition, in the same set of experiments, perturbation of *Zeb2* autoregulation via mutating the *Zeb2*-binding site in the promoter-proximal region of *Zeb2* results in strong downregulation of not only *Zeb2* but also *Tcf4*. This DNA segment, however, remains to be tested firmly for *Tcf4* occupancy. Preliminary observations suggest this signal is not strong, at least in NSCs, possibly as a result of the combination of *Zeb2* being expressed at a low level and *Tcf4* having a preference for enhancer binding, including its own enhancer located 200 kb within the gene^[78]. Thus, by extension, in NSCs and early neurogenesis, the aforementioned possibility of competition between *Zeb2* and a *Tcf4*-bHLH heterodimer for E-boxes is unlikely, although this still has to be investigated in depth for

competition at the *Zeb2* autoregulatory site.

Obviously, one still needs details on enhancers as well as meaningful SNPs for both *TCF4* and *ZEB2*, and how variation in those affects the levels of their mRNA and protein levels including in, e.g., human NPCs, as well as how this affects for each factor its interactions. In more general terms, this also means that, in neurodevelopmental iPSC-derived models, the effect of a perturbation of a given TF or chromatin remodeler will have to be documented in terms of its epistasis or GRN/PRN. This can occur via KD or, alternatively, via mutation in a domain or allelic genetic inactivation. The choice of KD vs. KO may influence epistatic relations and compensatory pathways, which may differ between choices. In any case, such sophisticated biochemical and functional studies are expected to confirm *ZEB2* and/or *TCF4* as important hubs in GRNs/PRNs, but also as modifier gene(s). They could very well be modifiers not only of one another (empowered by similarities of defects between MOWS and PTHS), but also of other causal genes in phenotypically overlapping neurodevelopmental syndromes (discussed in Ref.^[64]).

DIAGNOSIS AND STANDARD THERAPIES

A diagnosis of MOWS or PTHS is usually not made immediately after birth, but rather during infancy or childhood. It is based on thorough clinical evaluation, identification of characteristic physical features such as facial appearance (certainly for MOWS), and other symptoms, which do not generally present with all patients and, in the case of MOWS, clearly range from severe to milder cases. Of course, conditions of HSCR (for MOWS, but constipation is also very common in PTHS) and seizures (for both MOWS and PTHS) are also important indicators^[8,9,41,46]. In addition, for both syndromes, the structural and functional features become more pronounced with time; thus, diagnosis is easier in older patients. Checking for defects also includes imaging techniques^[45] such as computerized tomography scanning or magnetic resonance imaging of the brain and ultrasound of other organs (heart and kidney). Obviously, the clinical diagnosis is best confirmed by molecular genetic testing for mutations in the *ZEB2* and *TCF4* genes, respectively, with the current focus thus far on exon-sequencing, and it can be accompanied by the firm exclusion of other related disorders for which the causal genes (e.g., for ID) are known.

Currently, we can cure neither MOWS nor PTHS. The remarkably multiple functions of these TFs during embryogenesis and in the entire postnatal body, the knowledge of the precise regulation of *ZEB2* and *TCF4* levels in various cell types as concluded from studies of their gene expression control or via miRNAs (including lncRNAs, some of which act as sponges for, e.g., *ZEB2*-targeting miRNAs), and their complex interactome do not yet provide a basis solid and/or understood enough for drug-based or targeted protein-protein interaction-based intervention. Conversely, any attempt to increase *ZEB2* levels, which might be considered in MOWS (experimentally by gene- or RNA-based therapy), holds many dangers. For example, the resulting overproduction of *ZEB2* impacts on, e.g., pathological EMT (see the recent discussion^[139]) and correlates with bad prognosis in several epithelial-derived cancers, including melanoma^[103] and gastric cancer^[140].

The current treatment of MOWS and PTHS patients is directed toward the specific symptoms, and hence needs, of each affected individual and requires a team of specialists. These teams range from neurologists (prescribing anti-epileptic medication, which for example, in MOWS appears more easily managed in adolescents and adults) to gastroenterologists, dieticians, ophthalmologists, sometimes surgeons (in the case of HSCR in MOWS), pulmonologists (in PTHS, where the onset of breathing abnormalities can vary from months to years after birth), cardiologists, and psychologists to work together and case-by-case tailor the treatment plan. Treatment of bowel obstruction and HSCR involves complex surgery, often over several interventions, including colostomy and thereafter the removal of the non-functioning section of the colon

combined with rejoining the healthy segments of the bowel. Other surgeries may also be performed to treat specific congenital anomalies, such as heart defects.

Physical, occupational, and speech therapies are very useful in helping children with developmental delay to reach a higher potential, while coordinated establishment of patient registries, genetic mapping, and longitudinal follow-up of patients are very valuable. As argued here and elsewhere^[8], research in MOWS- and PTHS-like animal models (in particular, in challenged adult mice) in many biomedical fields is likely to inspire clinicians to follow up on their, e.g., injured (reduced pain sensitization, in MOWS) and aging patients more broadly, certainly in the case of complicating acute (including infections and immunity) or chronic (liver fibrosis^[102] and myelinating diseases^[51,99]) pathology.

CONCLUSION

It is amazing how investigation of mechanisms underlying monogenic syndromes, in particular by establishment of animal and cellular models combined with multi-omics approaches, has rapidly complemented ongoing parallel efforts in studies in clinical genetics, establishment of patient registries, and execution of genetic testing. At the same time, this combination of fundamental and clinical research provides insight into phenotypic overlap and possibly convergence between different syndromes. Here, we suggest this is the case for MOWS (*ZEB2*) and PTHS (*TCF4*), prompting us to summarize recent progress on both and combine them for the first time in this review. The progress is expected to provide a solid basis for designing next-generation cellular and animal models and use these to map transcriptomic signatures for each of the applied (including domain and patient-inspired) mutations of these TFs. We also highlight the importance of re-addressing their gene expression regulation (including via distal regulatory elements) and documenting and following up on patients with regard to non-neuronal cells of their nervous systems, as well as outside of these systems when these patients suffer certain acute or chronic pathologies.

DECLARATIONS

Acknowledgments

We thank all workers of the Center for Biomics-Genomics for sequencing and bio-informatics, Mike Dekker and Anne Korporaal for expert technical assistance, and Niels Galjart and Frank Grosveld for discussions. We are grateful to the clinical input from Livia Garavelli and Christiane Zweier.

Authors' contributions

Carried out most of the *TCF4* and *ZEB2* studies in the NPC models: Meert L, Birkhoff JC, Conidi A
Co-designed and supervised these projects: Poot RA, Huylebroeck D
All authors contributed to and approved the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Erasmus MC via departmental and BIG project funding (together with Erasmus University Rotterdam).

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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