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1 **TITLE**

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3 Mapping the molecular and cellular complexity of cortical malformations

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1 **ABSTRACT**

2 The cerebral cortex is an intricate structure underlying human features such as language and  
3 cognition. Cortical functions rely on specialized neurons, which emerge during development  
4 from complex molecular and cellular interactions. Neurodevelopmental disorders occur when  
5 one or several of these steps are incorrectly executed. While a number of causal genes and  
6 disease phenotypes have been identified, the sequence of events linking molecular disruption  
7 to clinical expression mostly remains obscure. Here, focusing on human malformations of  
8 cortical development, we illustrate how complex interactions at genetic, cellular and circuit  
9 levels combinatorially contribute to diversity and variability in disease phenotypes. Through  
10 select examples and an online resource, we propose that a multi-level assessment of disease  
11 processes is key to identify points of vulnerability and develop novel therapeutic strategies.

12

## 1 MAIN TEXT

2 The cerebral cortex, or neocortex, is critical to key behavioral functions in mammals, including  
3 language, sociability, and fine motor skills. This brain structure consists of several dozens of  
4 specialized types of neurons organized across layers and areas, which are generated during  
5 development by the divisions of diverse progenitor cells. Newborn neurons undergo sequential  
6 molecular programs that drive their specific local and long-range circuit connectivity and adult  
7 function (1). The complexity of corticogenesis is staggering and, while necessary for proper  
8 cortical function to emerge, the myriads of molecular and cellular developmental processes  
9 involved also provide multiple points of vulnerability for “malformations of cortical  
10 development” (2), here termed “cortical malformations”. These are usually first detected  
11 through their clinical expression, including developmental delay with various combinations of  
12 intellectual and motor disabilities, often associated with seizures. Despite the toll on patients  
13 and their caregivers, only limited treatments exist and while a number of causal genes have  
14 been identified, the sequence of events linking molecular disruption with clinical expression  
15 mostly remains obscure.

16 Here, to interrogate cortical malformations and highlight potential points of intervention,  
17 we first present basic principles of neocortical development and highlight vulnerable cellular  
18 compartments and processes, with particular focus on neurogenesis and neuron migration.  
19 Second, we present different “levels” of developmental processes, from genes and gene  
20 products to cells, circuits, and clinical expression. Focusing on cortical malformations, we  
21 illustrate how complex interactions within and across these levels may account for variable  
22 disease patterns. Finally, we propose a framework integrating these different levels of  
23 organization, towards better understanding and treatment of the disease, as well as an online  
24 resource (<http://genebrowser.unige.ch/humous/>) to identify and compare genetic and cellular  
25 developmental processes in mice, human embryos, and human organoids.

### 26 Neocortical development

27 During embryonic neocortical development, neurons are not born at the place where they will  
28 reside in adulthood, but instead migrate relatively long distances to reach their destination. Not  
29 only are cortical neurons born at different places, but distinct types of neurons are born at  
30 different times, over several days in mice (from embryonic day (E) 11 to 17 (3)) and several  
31 weeks in humans (from post-conception week (pcw) 8 to 24 (4)) (Fig. 1A). Generation of the  
32 cortex results from billions of cells undergoing four key sequential and partially overlapping

1 processes: (1) progenitor division and neurogenesis, (2) migration, (3) neuritogenesis (*i.e.*  
2 extension of axon and dendrites) and (4) synaptogenesis (Fig. 1A-C). Cortical malformations  
3 occur when one or several of these developmental events are improperly executed (Fig. 1B);  
4 they typically manifest as macroscopic anatomical defects visible with brain imaging  
5 techniques such as magnetic resonance imaging (MRI) (Fig. 2). Morphological manifestations  
6 include microcephaly (decreased brain size), lissencephaly (disrupted cortical folding and  
7 lamination), polymicrogyria (numerous small cortical folds), and heterotopias (abnormally  
8 positioned cells in periventricular or subcortical regions) (5). In other cases, more subtle defects  
9 are visible only in pathology specimens, *e.g.* in focal cortical dysplasias (abnormal cortical  
10 lamination) (2).

11 From E8.5-E9.5 in mice and pcw4 in human (4), progenitors of the dorsal pallium, at the  
12 rostral end of the neural tube, which initially form a single-layered neuroepithelium, undergo  
13 self-replicating divisions to increase in numbers before generating apical radial glia (aRG, also  
14 called apical progenitors) and forming an expanded germinal zone called the ventricular zone  
15 (3). Around E11.5 in mice and pcw8 in human, aRGs start dividing asymmetrically to give rise  
16 to their first neuronal progeny (Fig.1A) (4). Different types of excitatory cortical neurons are  
17 then sequentially born between E11.5 and E17.5 in mice (pcw8-24 in human) and distribute to  
18 form six radially-organized layers in an inside-out manner: deep layer neurons, which project  
19 subcortically, are born first while superficial layer neurons, which project intracortically, are  
20 born last (Fig. 1A) (1). Of note, inhibitory GABAergic neurons, which are not discussed in  
21 detail in this review, are born in a distinct germinal zone, the ventral pallium, and migrate at  
22 later timepoints into the cortex to form local circuits with glutamatergic neurons (6).

23 *Radial cortical organization* – The laminar organization of the adult neocortex tightly relies  
24 on the radial polarity of progenitors and their daughter cells during development. Accordingly,  
25 aRGs are highly polarized cells with a radial process extending to the pial surface, necessary  
26 to guide the daughter neuron migration (Fig. 1A). Adherens junctions at the apical (*i.e.* towards  
27 the ventricle) pole of aRGs are critical to this polarity, as they maintain a cohesive ventricular  
28 zone and regulate the balance between proliferation and differentiation through cell-cell  
29 interactions (3). Mutations of genes coding for adherens junction proteins, such as  
30  $\alpha/\beta$ -catenins and N-cadherin, indirectly disrupt cell divisions, leading to changes in cortical  
31 size and folding, as well as neuronal heterotopias in mice and human (7-10). Similarly,  
32 mutations in the genes coding for cell-cell contact proteins such as DCHS1 and FAT4 disrupt  
33 aRG polarity and neuronal migration, resulting in human periventricular heterotopias (11).

1 Another notable determinant of aRG polarity is the primary cilium, a specialized organelle  
2 which transduces signals from ventricular cerebrospinal fluid (Fig. 1A) (3). Mutations in genes  
3 coding for cilium proteins (“ciliopathy” genes, *e.g.* *BBS1*, *BBS7*, *BBS10* and *TMEM216*) affect  
4 progenitor proliferation and newborn neuron migration in mice (12) and mutations in the  
5 microtubule-associated protein encoding gene *EML1*, which causes neuronal heterotopia in  
6 mice and humans, are likewise thought to act by destabilization of the primary cilium (13, 14).

7 *Cycling of progenitor cells* – The ability to undergo cell cycle is a central feature of progenitors.  
8 A number of mutations can perturb this process, either by affecting cell divisions, or through  
9 premature differentiation into neurons. For example, mutations of genes coding for proteins  
10 controlling mitotic spindle organization (Fig. 1A) typically lead to premature differentiation  
11 and / or aRG apoptosis, resulting in reduced neurogenesis, and manifesting in humans as  
12 microcephaly with or without lissencephaly (*e.g.* *ASPM*, *CENPJ*, *WDR62* (15)). Of note, both  
13 premature differentiation into neurons as well as excessive self-replication of progenitors may  
14 in principle both result in decreased neuronal output: in one case the progenitor pool is  
15 prematurely depleted while in the other, progenitors fail to give rise to neurons. Divisions of  
16 aRGs are initially rapid and then slow down as corticogenesis unfolds (from 8 hours at early  
17 stages to 18 hours at the end of corticogenesis in mice, and from 23 to 54 hours in primates  
18 (16, 17)). The slowing down of cell cycle as corticogenesis proceeds may *per se* confer new  
19 properties to daughter cells as low-affinity DNA ligands such as transcription factors are more  
20 likely to bind when target sites are available for longer times. Hence, disruption of cell cycle  
21 not only affects the number of neurons being generated (typically decreasing neuronal output)  
22 but also the relative proportions of daughter neuron types, since distinct types are sequentially  
23 produced at different stages of corticogenesis (18).

24 *Diversity of progenitor cells* – aRGs not only generate neurons, but also a distinct type of  
25 progenitor, termed intermediate progenitors (also called basal progenitors), which delaminate  
26 from the ventricular zone and form a new germinal layer, the subventricular zone (Fig. 1A).  
27 Intermediate progenitors are more neurogenic than aRGs, and act to boost neuronal production  
28 (19). Consistent with such a function, human mutations affecting intermediate progenitors,  
29 such as mutations in *EOMES* (also known as *TBR2*), perturb gyri formation and result in  
30 microcephaly, lissencephaly or polymicrogyria (20). In most gyrencephalic species (*i.e.*  
31 species with a folded neocortex), including primates, additional progenitor types exist,  
32 including basal radial glia (also called outer radial glia), which form a distinct proliferative  
33 sub-compartment, the outer subventricular zone (Fig. 1A). Basal radial glia express distinctive

1 genes (*e.g.* *TMEM14B* (21)) and morphologies (*e.g.* many have a basal process but no apical  
2 process), and are thought to underlie the disproportionate expansion in superficial layers  
3 occurring during gyrification (22).

4 *Neuronal migration* – Following cell division, while undergoing a complex series of  
5 morphological changes, postmitotic daughter neurons migrate radially along aRG basal  
6 processes away from the ventricular zone to the cortical plate, where they settle in their  
7 appropriate final positions. The radial migration of neurons relies on the extension of a leading  
8 process, with the leading edge responding to environmental cues. The neuron’s centrosome  
9 moves within the leading process allowing somal translocation and locomotion (Fig. 1A) (23).  
10 The proper execution of this migratory sequence is key to building the correct cortical  
11 architecture and relies tightly on microtubule and actin cytoskeleton-associated proteins.  
12 Accordingly, mutations in the genes coding for these proteins have dramatic effects on cortical  
13 structure (5, 15), and account for a disproportionate fraction of cortical malformations, as will  
14 be detailed in a later section.

15 Preplate splitting, which occurs at E11 in mouse (human pcw9-10) (24), is a critical event  
16 during corticogenesis and evolution (Fig. 1A). The glycoprotein Reelin secreted by Cajal-  
17 Retzius cells present in the marginal zone plays an important role in these processes. Preplate  
18 splitting defects consistently give rise to abnormal cortex development due to aberrant cortical  
19 neuron position. Indeed, lamination defects in *Reeler* mice, mutant for the *Reln* gene, not only  
20 reflect abnormal neuronal migration, but also abnormal splitting of the preplate into a  
21 superficial marginal zone (which becomes layer 1) and a transient deep layer subplate  
22 (sometimes called layer 6b) (Fig. 1A). In humans, mutations in *RELN* cause autosomal  
23 recessive lissencephaly (25). As discussed in a later section, despite the severe cortical  
24 lamination defects in *Reeler* mouse mutants, long-range cortical input/output connectivity  
25 appears largely preserved (26, 27). *Pomgnt2* mouse mutants, a model of “cobblestone”  
26 lissencephaly caused by mutation in the *POMGNT2* gene in human, also show preplate splitting  
27 defects, since abnormal clusters of subplate neurons are observed in superficial layers (28).  
28 The subplate itself plays an important role in the radial migration of the neurons which traverse  
29 it and, later, in circuit assembly: subplate neurons make transient synaptic connections with  
30 migrating neurons, which primes their radial migration (29), and also act as a scaffold for  
31 thalamocortical and inhibitory circuit maturation (30, 31). Although mutations specifically  
32 affecting this process have not been reported in humans, the subplate is usually visible in

1 human fetal MRIs; in the future, it may be possible to reveal subtle subplate defects underlying  
2 more significant circuit dysfunction.

3 *Building circuits* – Neuritogenesis and synaptogenesis are critical for circuit assembly and a  
4 bottleneck for cortical function. They have been discussed in detail in recent reviews (32) and  
5 will only be briefly covered here. Axon-dendrite polarization starts soon after neurons are born,  
6 when they still are migrating (33). Axon and dendrite growth relies on their growth cones which  
7 are highly dynamic actin-rich protrusions sensitive to environmental guidance cues (Fig. 1A).  
8 As is the case for migration, axonal guidance and extension thus heavily rely on cytoskeletal  
9 function. Cortical malformations can be accompanied by anomalies in axonal tracts, most  
10 notably in the form of atrophy or agenesis the corpus callosum, which connects both  
11 hemispheres in human (34). Other anatomically less prominent axonal pathway defects are  
12 likely to occur, and the use of MRI techniques such as diffusion tensor imaging (35) may in  
13 the future further address this possibility. Synaptic dysfunction, including inhibitory synaptic  
14 dysfunction, is thought to underlie many if not most neuropsychiatric disorders in humans (36),  
15 and like migration and neuritogenesis, synaptogenesis involves a highly organized  
16 cytoskeleton (Fig. 1A). Neuropsychiatric disorders are not generally associated with salient  
17 MRI defects, yet some level of anatomical dysfunction likely exists. For example, patches of  
18 cortical disorganization and white matter defects (particularly in the corpus callosum) have  
19 been reported in patients with autism spectrum disorder (37, 38). Similarly, decreased neuronal  
20 density have been reported in neuropathological specimens of patients with schizophrenia (39).  
21 The extent to which these anatomical findings drive the clinical phenotype or are secondary to  
22 another underlying process remains unclear.

### 23 **Sources of complexity in cortical malformations**

24 Cortical malformations have a broad range of clinical expression, including seizures,  
25 intellectual disabilities, autistic features, failure to reach developmental milestones, and non-  
26 neurological co-morbidities (e.g. dysmorphic facial and musculoskeletal features, skin  
27 abnormalities, cardiovascular defects) (5). These features alone often do not allow  
28 identification of the cause of the disease, because genetically diverse defects lead to largely  
29 overlapping clinical pictures, and impairment of single genes can lead to multiple clinical  
30 defects. Thus, while an increasing number of causal genes is being identified and a diversity of  
31 anatomical and clinical phenotypes have been recognized (15), the sequence of events linking  
32 molecular disruption to final outcome remains mostly out of reach. In the following section,  
33 we will highlight potential sources of variability which have precluded establishment of clear

1 causal relationships across genes and molecules, cell types and circuits, and clinical expression  
2 (Fig. 3A).

3 *Genetic and molecular dysfunction* – Neurodevelopmental disorders can result from single  
4 point mutations in critical genes (*i.e.* monogenic mutations; Fig. 3B), or from DNA sequence  
5 variations at multiple loci that together increase disease probability. Convergent, divergent, or  
6 mixed relationships between genetic defect(s) and disease phenotype(s) may occur (Fig. 3C-  
7 F), and penetrance of the genetic mutation may be incomplete and variable (*i.e.* the mutation  
8 is not expressed at the clinical level (Fig. 3G)), together complexifying causal analyses.  
9 Understanding this cross-level organization is critical in therapeutic terms: for example,  
10 “hidden hubs” represent convergent points of pharmacological intervention across disease  
11 phenotypes, while disorders with “hidden complexity” would require distinct treatments  
12 despite similar clinical presentations (Fig. 3E, F). The contribution of monogenic causes varies  
13 across conditions; in the case of lissencephaly for example, 20% of cases could not be  
14 associated with a single genetic cause (40), which may reflect yet un-identified gene mutations,  
15 mutations in non-coding regions (41), polygenic or multifactorial inheritance, modulated by  
16 the influence of non-genetic factors (*e.g.* infectious or toxic), or somatic mosaicism (42). While  
17 monogenic causes appear to predominate in cortical malformations, neuropsychiatric disorders  
18 such as autism spectrum disorders and schizophrenia instead appear to mostly involve  
19 polygenic processes including through chromosomal deletions (39). Of note, in chromosomal  
20 deletions, disease-causing genes need not to be in the affected chromosomal region as local  
21 DNA conformation changes may in principle epigenetically alter gene accessibility in other  
22 parts of the genome. Although not discussed in detail here, some genetic mutations occur in a  
23 clonal manner, *i.e.* do not affect the whole brain but only the progeny of some cells with  
24 somatic mutations. Somatic mutations have been clearly established as a cause of focal cortical  
25 dysplasia and hemi-megalencephaly (hypertrophy of a single hemisphere) (42), but whether  
26 clonal processes are involved in other disorders, and in particular whether at least some  
27 psychiatric disorders may result from mosaic synaptic dysfunction, is unknown (43).  
28 Overlapping clinical pictures can emerge from mutations in genes which are not apparently  
29 functionally correlated. For example, over 200 *de novo* variants have been identified in a cohort  
30 of patients with periventricular heterotopia, suggesting that many genes will be associated with  
31 this condition, with a high level of convergence in the final outcome (*e.g.* mismigration in the  
32 case of heterotopias) (44).

1       Complexifying the situation, the aforementioned developmental processes (*i.e.*  
2 neurogenesis, migration, axo- and synaptogenesis) often involve redundant molecular  
3 mechanisms. For example, the cyclin-dependent kinase CDK5 (whose gene mutation is  
4 associated with lissencephaly in human (45)) is critical for mitosis in mouse progenitors, but  
5 also in their daughter neurons for migration and gyrification, synapse formation and even long-  
6 term plasticity in adults (Fig. 1C) (46, 47). Similarly, many, if not most, axon guidance  
7 molecules also have earlier roles in neuron migration and even neuron generation, and/or in  
8 synapse and spine formation and maturation (48-50). For example, the Ephrin receptor EphB2  
9 both guides corpus callosum axons and stabilizes synaptic contacts (51, 52). Gene mutations  
10 may therefore affect sequential cellular processes in parallel. Such versatility in protein  
11 function likely contributes to the complexity and variability of disease processes.

12 *Cytoskeletal perturbations* – Given their involvement in multiple cellular processes, from  
13 cytokinesis to migration and neuritogenesis, mutations in genes coding for cytoskeletal proteins  
14 are particularly prevalent causes of cortical malformations. Strikingly however, quite different  
15 anatomical defects can result from mutations in genes coding for apparently related proteins.  
16 For example, in “tubulinopathies”, mutations in a tubulin-encoding gene, *TUBA1A*, generally  
17 decreases gyrification in humans, while disruption of another tubulin, *TUBB3* often gives  
18 polymicrogyria (53). Microtubule-related proteins can behave similarly, as mutations in the  
19 gene coding for doublecortin (*DCX*, which stabilizes microtubules and regulates certain  
20 molecular motors during neuronal migration (54)), or *LIS1* (which regulates dynein and is  
21 required for interkinetic migration of aRGs as well as neuronal migration (55)), variably result  
22 in either subcortical heterotopias or lissencephaly in humans (5). In the case of *DCX*, which is  
23 located on the X chromosome, mutations in women typically lead to heterotopia while in men  
24 it is associated with lissencephaly, suggesting dose-dependent effects (56).

25       Post-transcriptional and post-translational dysregulations are also a cause of cortical  
26 malformations. For example, abnormal alternative splicing can occur in the Filamin A actin-  
27 binding transcript (*Flna*), causing periventricular heterotopias in humans (57). Inhibition of a  
28 neuronal “poison exon” by the Polypyrimidine Tract Binding Protein PTBP1 is necessary to  
29 maintain neural progenitor identity. In the absence of PTBP1 function, de-repression of this  
30 poison exon leads to decreased expression of *FLNA* and precocious neuronal differentiation  
31 (Fig. 4A). Mutations of proteins modifying the conformation and activity of other proteins,  
32 like kinases (*e.g.* PI<sub>3</sub>K/AKT pathway (58)), glycosylases (59) and tubulin chaperones /

1 modifiers (*e.g.* Tubulin Folding Cofactor D (*TBCD*) (60)) are further post-translational causes  
2 of cortical malformations.

3 Obviously, not all cortical malformations are the result of disrupted cytoskeletal function.  
4 Other classes of proteins, such as transcription factors, are also involved (*e.g.* ARX, PAX6,  
5 EOMES and FOXP1 whose gene mutations lead to severe cortical malformations in humans  
6 (Fig. 2) and mice (20, 61-63)). Such examples are, however, comparatively rare despite their  
7 critical function in orchestrating developmental genetic programs. One explanation for this  
8 apparent paradox is that many transcription factors may be “too” critical for corticogenesis,  
9 such that loss of their function is embryonically lethal.

10 *Disrupted spatio-temporal choreographies* – Normal gene expression is tightly regulated in  
11 space and time during development. Hence, abnormal dynamic regulation of gene expression  
12 is likely a critical factor in the pathogenesis of cortical malformations. Supporting this  
13 possibility, *Flna* mutant mice do not display neuronal migration defects, while acute RNAi-  
14 mediated knockdown of *Flna* in rats reproduces the periventricular heterotopias similar to those  
15 observed in human patients (64). Also, constitutive *Dcx* mutant mice have a fairly normal  
16 neocortex and behavior (65), while acute RNAi-mediated knockdown of *Dcx* causes  
17 subcortical band heterotopia (66) (Fig. 4B). In principle, while dysfunction of early-onset genes  
18 cascade onto a broad array of subsequent cellular processes and may lead to more severe  
19 phenotypes, early-onset defects may also be more easily compensated for than defects affecting  
20 late differentiation stages.

21 Spatial regulation of gene expression is also important, particularly in the cortex where  
22 functionally specialized areas exist. In many cases, malformations are focal or regionalized  
23 rather than diffuse, although what determines their location is usually unknown. Cortical  
24 malformations often show antero-posterior gradients (40). For example, *FLNA* mutations  
25 typically cause frontal-predominant heterotopias (67), and *GPR56* mutations frontal-  
26 predominant polymicrogyria (68). In contrast, *CEP85L* shows higher expression in posterior  
27 cortical regions and mutations in this gene lead to posterior-predominant polymicrogyria (69).  
28 Some affected genes, however, are ubiquitously expressed (*e.g.* *GPR56* (70)); interactions with  
29 locally expressed binding partners or specific developmental dynamics may here account for  
30 the stereotypic location of the cortical defect. In some cases, such as *FLNA*, *GPR56* or *EOMES*,  
31 spatial restriction of gene expression in regions underlying gyri or sulci (71) could further  
32 contribute to impairments in cortical folding. Finally, somatic mutations may also account for

1 the focal nature of a defect (*e.g.* PI<sub>3</sub>K pathway (72)), although what determines area location  
2 is often unknown (42).

3 *Abnormal cell types* – Cell types are the end-result of the dynamic spatio-temporal expression  
4 of genes during development. Cortical malformations typically affect multiple types of neurons  
5 and/or progenitors, which is in contrast with neurodegenerative diseases, where single cell-  
6 types (*e.g.* dopaminergic neurons in Parkinson disease) are predominantly affected. The  
7 apparent lack of cell type specific impairments may reflect that differentiation processes are  
8 mostly generic across neuron types, and that cell type identity mostly emerges from the  
9 combinatorial interactions of multiple genes rather than single master regulators (73). In some  
10 cases (mainly focal cortical dysplasia, autism spectrum disorder, schizophrenia and epilepsy),  
11 specific defects have also been reported in inhibitory neurons, astrocytes and oligodendrocytes  
12 (43, 74) (Fig. 4C). Failure in astrocyte-dependent removal of extracellular debris may lead to  
13 a chronically pro-inflammatory environment, potentially increasing susceptibility to disease,  
14 but the extent to which reported changes in non-neuronal cells are reactive to neuronal loss is,  
15 however, often unclear (75). Of note, since astrocytes are born towards the end of neurogenesis  
16 (likely from the progeny of aRGs), mutations that affect aRG divisions are probably associated  
17 with a decreased number of astrocytes, although this has not been systematically assessed.

18 Depending on progenitor types and stage of corticogenesis, daughter neurons differ and the  
19 aberrant generation of one of these progenitor types could in principle affect specific progenies.  
20 For example, mutations affecting intermediate progenitor function could preferentially affect  
21 superficial layers, since such progenitors become more and more prevalent as corticogenesis  
22 unfolds and superficial layer neurons are born last. Recently, electrophysiological properties  
23 of progenitors have been shown to contribute to the sequential generation of neuronal types  
24 (76) and may thus contribute to cortical malformations. Accordingly, mutation in the sodium  
25 channel Nav1.3 (SCN3A) gene affects outer subventricular zone progenitors and causes focal  
26 polymicrogyria in humans, potentially reflecting excess or altered superficial neuron  
27 production (77).

28 *Abnormal circuits* – Although neuronal heterotopia likely affects circuit assembly, neuronal  
29 identity and basic circuit properties are often conserved despite abnormal neuronal position. In  
30 mice, ectopic neurons usually still express their proper laminar markers and display conserved  
31 long-range projections (27, 78, 79) (Fig. 4D). Similarly, L5-type neurons in L4 retain their  
32 connectivity, as do L4-type neurons in L2/3 (80, 81) and ectopic neurons still attract the proper  
33 subtypes of GABAergic interneurons with whom they make synaptic connections (as if they

1 were normally positioned in the cortex), suggesting that local connectivity might likewise be  
2 preserved (82). How GABAergic interneuron migration is affected in microcephaly or other  
3 cortical malformations has not been systematically examined (but see (83)), but disruption of  
4 this process could provide a cellular/circuit explanation for the seizures seen in some patients.  
5 In the *Reeler* mouse, although distinct layers are no longer visible, L4-type neurons still receive  
6 proper innervation from the thalamus and somatotopic innervation of the cortex is relatively  
7 preserved (26, 27) (Fig. 4D). Supporting spared circuit function, perception, learning and  
8 memory in *Reeler* mutant mice are little affected by disrupted cortical lamination (27), although  
9 more cortex-targeted behavioral tests would likely reveal some level of dysfunction. Together,  
10 these results suggest that neuron identity emerges largely cell-autonomously, such that long-  
11 distance projections (and possibly microcircuits) may be relatively spared in neuronal  
12 heterotopias.

13 *Behavior and clinical features* – As a consequence of the number and complexity of the  
14 molecular and cellular processes involved in cortical disorders, the clinical manifestations are  
15 heterogeneous, and include intellectual disabilities, epilepsy, and a broad spectrum of  
16 neuropsychiatric manifestations. What determines this spectrum is unknown, but postnatal,  
17 environmental factors and overall genetic background may contribute (84). Epilepsy often  
18 occurs in the context of cortical malformations, but which factors determine seizure  
19 susceptibility is unclear. In mouse models of cortical heterotopia, the ectopic and isotopic  
20 cortices remain interconnected, which may contribute to abnormal neuronal activation and  
21 seizures seen in human patients (85, 86). In some cases, abnormal electrical activity was  
22 initiated within or adjacent to the heterotopia while in others, seizures were initiated from the  
23 overlying cortex. This suggests either that aberrantly located neurons are capable of generating  
24 dysfunctional circuits at a distance, or that the overlying homotopic cortex is abnormal (87).  
25 Like other clinical features, intellectual functions are also variably affected, spanning from  
26 essentially normal functions to severe disability, in which case seizures are often associated  
27 (88). In the absence of additional biomarkers, this relatively generic array of symptoms and  
28 signs, which have historically been the main criterion for disease classification, thus offer  
29 limited opportunities for a mechanistic understanding of the disease process.

### 30 **Towards a multi-level assessment of neurodevelopmental disorders**

31 The examples above highlight several levels (*i.e.* genetic, molecular, cellular, circuit and  
32 behavioral) within and across which combinatorial interactions may occur during cortical  
33 malformations and preclude a causal understanding of the disease process (Figs. 3-4).

1 Understanding the processes at play at each of these levels for individuals is key to provide  
2 patients and their families with prognostic indicators (including biomarkers and, more broadly,  
3 recognized “endophenotypes”) and, in the longer term, therapeutic perspectives.

4 Towards these two aims, the use of animal models, including mouse, ferret and non-human  
5 primates (the latter being gyrencephalic and thus closer to humans) is important, in particular,  
6 at some point, to assess the therapeutic effect of approaches on behavior, including through  
7 insertion of human mutations via gene editing technologies (47). *In vitro* human models are  
8 also emerging as a promising and feasible avenue: somatic cells can now be reprogrammed  
9 into induced pluripotent stem cells (iPSCs) and serve as a basis for the generation of 3D  
10 organoids and assembloids of the brain (89, 90). Organoids have been used to study a variety  
11 of neurodevelopmental disorders including cortical malformations (*e.g.* *CDK5RAP2* mutation  
12 leading to cobblestone lissencephaly in humans) (91). Despite their limitations in terms of  
13 reproducibility and restriction to early stages of corticogenesis (gyrification is for example  
14 challenging to study in these models) (92), organoids are poised to become broadly used in  
15 personalized medicine by giving access to the neurons of the patient under study.

16 Recent advances in single-cell transcriptomics now allow the dynamic developmental  
17 expression of genes in emerging cell types to be assessed (93, 94) (Fig. 5A), potentially linking  
18 cellular diversity and characteristics with circuits and behavioral repertoires. Comparison of  
19 gene expression across brain development in mouse, monkey, human and primate- / human-  
20 derived brain organoids is particularly important to identify perturbed processes and select the  
21 appropriate study model. As a first step in this direction, here we provide an online resource  
22 (<http://genebrowser.unige.ch/humous/>) compiling transcriptional maps across development  
23 and neuron differentiation for mouse embryos, human embryos and human-derived organoids  
24 (94-96). As an example of an application of such a database, combinatorial analysis of arrays  
25 of genes implicated in microcephaly, lissencephaly or polymicrogyria using this resource  
26 confirms that microcephaly predominantly affects progenitor function, while lissencephaly and  
27 polymicrogyria affect postmitotic neurons (Fig. 5B). In the latter two cases, human data  
28 emphasizes that progenitors may also be affected, highlighting the value of *trans*-species  
29 comparisons (see arrowheads in Fig. 5B).

30 Multi-omics approaches, in which transcriptomics are combined with genomics,  
31 epigenomics and proteomics will provide an opportunity to further unravel the cell type  
32 specific processes at play and distinguish core molecular / cellular disease processes from  
33 idiosyncratic or stochastic variability. Comparing the distribution of affected processes (at any

1 level of analysis) across patients will help to build a spectrum of abnormal states and predict  
2 vulnerable processes (Fig. 5C). Integrative approaches including electrophysiological,  
3 imaging, and clinical and biological data from patients, for example using state-of-the art  
4 artificial intelligence algorithms (97, 98), may allow the bridging of DNA mutation(s) to gene  
5 expression, cellular, anatomy and circuit consequences (Fig. 5D). This will be instrumental for  
6 a pathogenic classification of diseases, an essential step for a more precise patient  
7 stratification and for the design of personalized diagnostic and therapeutic tools. Although this  
8 level of integration may seem futuristic, high-throughput techniques and analytical tools are  
9 increasingly available, paving the road for such strategies in a realistically close future.

10  
11  
12

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4

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15 and designed the figures.

16 **Competing interests:** The authors declare no competing interests.

17 **Data and materials availability:** In Fig. 5, data from mouse embryos are from (94); data  
18 from human embryos and human-derived organoids are from (95, 96).

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20

## 1 **FIGURE LEGENDS**

2 **Fig. 1: Key steps of corticogenesis and their susceptibility to disease.** (A) Summary of  
3 embryonic corticogenesis. One neuron can be followed from its birth at the ventricular zone,  
4 to its migration toward the cortical plate, and then its maturation (arrow). The role of  
5 cytoskeletal processes at each of these steps is highlighted on the right. (B) Literature-based  
6 summary of knowledge on the involvement of these developmental steps in specific  
7 neurodevelopmental disorders. The numbers of publications were found using the text mining  
8 EasyPubMed R package, which automatically identifies publications containing given the  
9 combinations of keywords indicated (*e.g.* “microcephaly” and “proliferation”). These numbers  
10 were then normalized for number of publications for each of the developmental steps and each  
11 of the disorders. The dendrogram represents unbiased clustering of the diseases based on this  
12 data. (C) Overlapping cellular processes and pleiotropic molecular mechanisms involving  
13 CDK5 across development and in the adult brain. aRG, apical radial glia; bRG, basal radial  
14 glia; CP, cortical plate; E, embryonic day; IN, interneuron; IPC, intermediate progenitor cell;  
15 N, excitatory neuron; pcw; post-conception week; SVZ, subventricular zone; VZ, ventricular  
16 zone; WM, white matter.

17

18 **Fig. 2: Cellular mechanisms at play in human cortical malformations.** Cells with abnormal  
19 developmental trajectories are in pink. Reduced progenitor proliferation (sometimes associated  
20 with abnormal neuron migration) leads to microcephaly. Abnormal neuron position can lead  
21 to agyria and lissencephaly (reduced migration and/or progenitor misposition), polymicrogyria  
22 (increased migration and/or excess of basal radial glia), cobblestone lissencephaly (excessive  
23 migration), periventricular heterotopia (neurons stay close to the ventricle) or subcortical band  
24 heterotopia (neurons stay below the cortex). In focal cortical dysplasia, clonal mutations affect  
25 subpopulations of mature cells, which become dysmorphic (balloon cells, dysmorphic  
26 neurons). Genes mentioned in this review are listed (bold: genes with published human-derived  
27 brain organoid models, \*: gene studied in the ferret, green: genes coding for transcription  
28 factors). Pink arrowheads on MRIs highlight the malformations. MRI illustrations are from  
29 (15) and (2) for polymicrogyria and focal cortical dysplasia (Reprinted from *Semin Cell Dev*  
30 *Biol* vol 76, D. M. Romero, N. Bahi-Buisson & F. Francis, Genetics and mechanisms leading  
31 to human cortical malformations, pages 33-75, 2018, with permission from Elsevier;  
32 Definitions and classification of malformations of cortical development: practical guidelines;  
33 M. Severino et al., *Brain* 2020; awaa174, with permission from Oxford University Press).

1

2 **Fig. 3: From gene mutations to diseases: levels of complexity.** (A) Levels of organization  
3 during corticogenesis, from DNA (*i.e.* genes, represented by letters) to RNA and proteins (*i.e.*  
4 gene expression), to cells, circuit and anatomy, and phenotype. Each circle represents a given  
5 feature of that level (*e.g.* a gene, a protein, a circuit). Interactions within levels are linked  
6 through complex relationships (dashed lines) to states at other levels (black lines). (B-G)  
7 Examples of abnormal feature relationships across levels in disease (highlighted in pink). In  
8 **B-D**, linear (monogenic), convergent (polygenic) and divergent relationships between genetic  
9 defects and phenotype(s) are illustrated. (E) Hidden complexity: note that from a gene-  
10 phenotype point of view, this relationship appears linear. (F) Hidden hub: different genes may  
11 lead to distinct phenotypes *via* hidden feature hubs influencing several downstream pathways.  
12 (G) Incomplete penetrance: not all genetic features are expressed at other levels. In A, source  
13 images for human brain section and phenotype are from <https://msu.edu/~brains/brains/> and  
14 ©biorender.com, respectively.

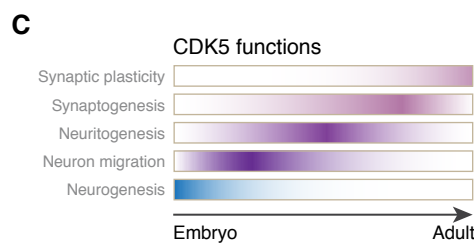
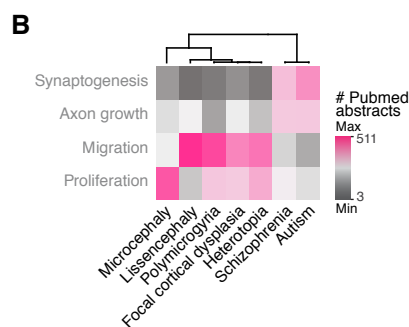
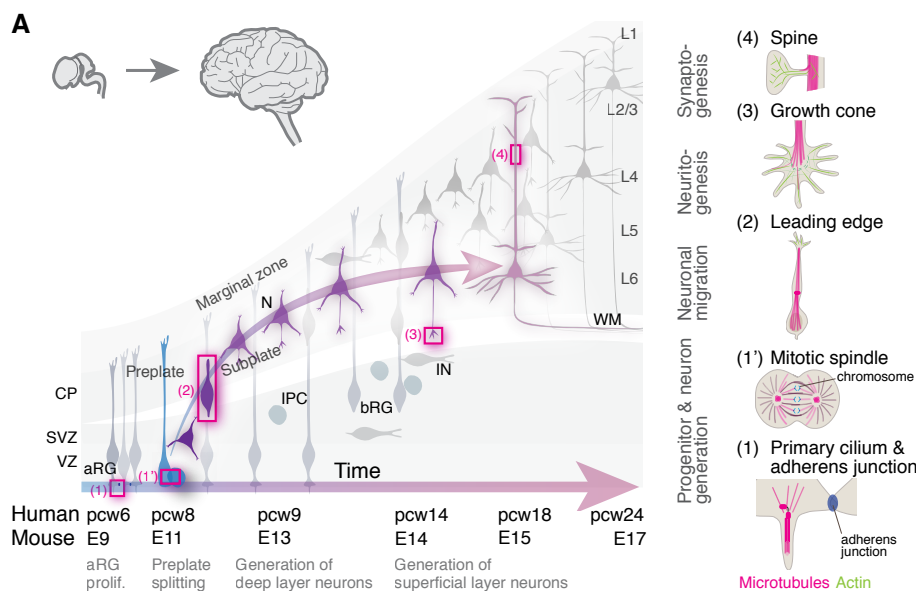
15

16 **Fig. 4: Spatiotemporal and cell type regulations of gene expression and malformations.**  
17 (A) Alternative splicing of *FLNA* gene regulates its role in proliferation *versus* neuron  
18 differentiation of cortical progenitors (top). Des-inhibition of the *FLNA* poison exon leads to  
19 precocious neuron differentiation and periventricular heterotopia in humans (bottom, pink  
20 arrowheads). (B) *DCX* mutation in human is associated with subcortical band heterotopia (pink  
21 arrowheads). In mice, constitutive *Dcx* loss of function does not cause major cortical defects,  
22 while acute uni/bilateral loss of function induces large subcortical heterotopia. Heterotopia are  
23 highlighted in pink. KO, knock-out. (C) The numbers of publications containing the indicated  
24 combinations of keywords (cell types and neurodevelopmental diseases) were identified with  
25 the EasyPubMed R package as described in Fig. 1. (D) Basic connectivity rules in the mouse  
26 somatosensory cortex. This connectivity appears largely conserved when neurons are  
27 mispositioned, like in the “scrambled” cortex of *Reeler* mice or in case of heterotopia. ASD,  
28 autism spectrum disorder; FCD, focal cortical dysplasia; HET, heterotopia; L, layer; LIS,  
29 lissencephaly; MIC, microcephaly; PMG, polymicrogyria; SZ., schizophrenia. In B, source  
30 image for mouse is from ©biorender.com.

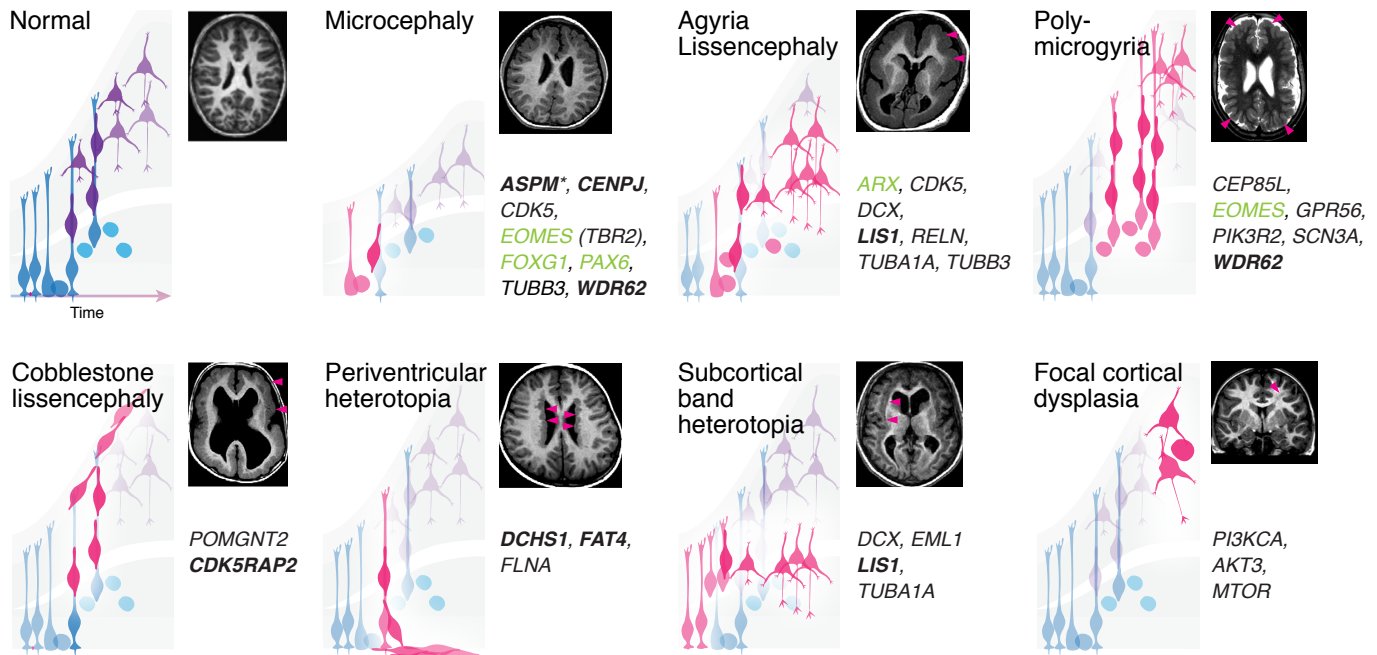
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1 **Fig. 5: Multimodal assessment of neurodevelopmental disorders.** (A) Identification of  
2 developmental gene dynamics in specific cell types through single-cell RNA sequencing (top).  
3 Illustration from the online resource <http://genebrowser.unige.ch/humous/> comparing data  
4 from mouse, human embryos, and human derived- brain organoids (bottom). (B) Average  
5 expression of genes associated with microcephaly (MIC), lissencephaly (LIS) and  
6 polymicrogyria (PMG) in mouse, human embryos, and human derived- brain organoids.  
7 Arrowheads show human-specific patterns. (C) Schematic representation of the probabilistic  
8 distribution of affected processes in sample disorders. Pink cubes define abnormal states. (D)  
9 Hypothetical patient classification through integration of multiple datasets. Here patients were  
10 clustered into 2 groups based on the integration of several features, from genes to clinical  
11 expression. ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput  
12 sequencing) allows to identify chromatin accessible regions. Mass-spectrometry and co-  
13 immunoprecipitation allow to identified proteins based on their molecular weight and  
14 interaction(s) between proteins, respectively. *In vitro* and animal model data bring  
15 complementary information about the cellular effect(s) of a gene mutation. aRG, apical radial  
16 glia; bRG, basal radial glia; CSF, cerebrospinal fluid; fMRI, functional magnetic resonance  
17 imaging; iN, immature neurons; IPC, intermediate progenitor cells; MCD, malformations of  
18 cortical development; mN mature neurons. In B, data from mouse embryos are from (94); data  
19 from human embryos and human-derived organoids are from (95, 96). The 2-dimensional  
20 landscapes of gene expression across time and differentiation were performed as described in  
21 (94). In B, source images for mouse and human are from ©biorender.com.

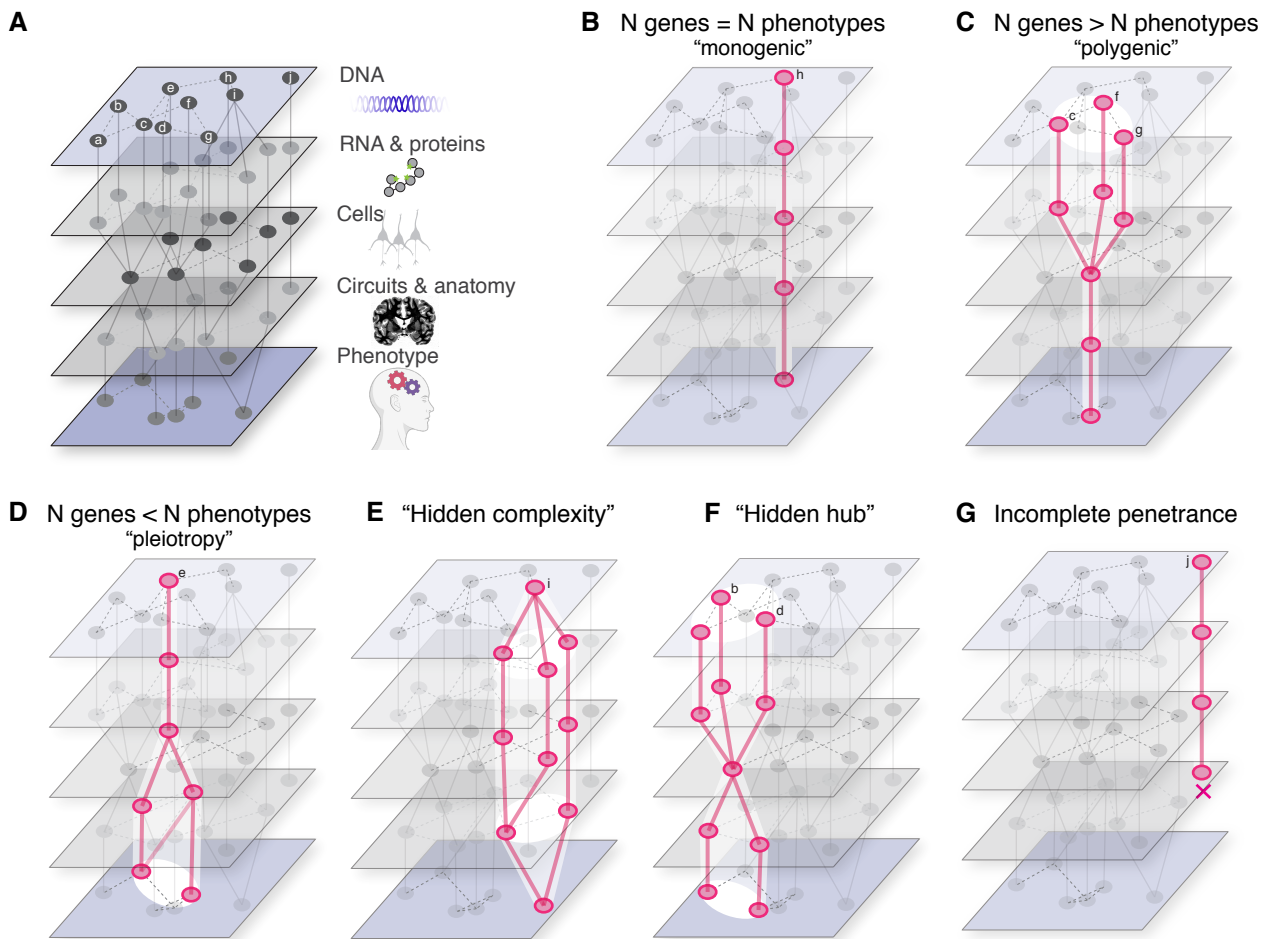
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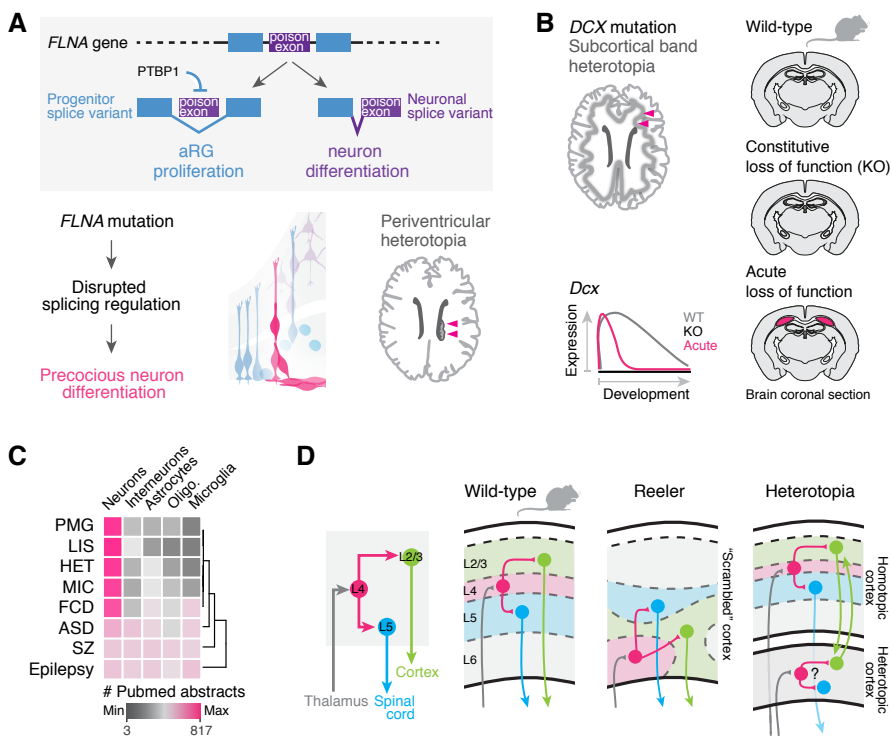
**Fig. 1: Key steps of corticogenesis and their susceptibility to disease. (A)** Summary of embryonic corticogenesis. One neuron can be followed from its birth at the ventricular zone, to its migration toward the cortical plate, and then its maturation (arrow). The role of cytoskeletal processes at each of these steps is highlighted on the right. **(B)** Literature-based summary of knowledge on the involvement of these developmental steps in specific neurodevelopmental disorders. The numbers of publications were found using the text mining EasyPubMed R package, which automatically identifies publications containing given the combinations of keywords indicated (e.g. “microcephaly” and “proliferation”). These numbers were then normalized for number of publications for each of the developmental steps and each of the disorders. The dendrogram represents unbiased clustering of the diseases based on this data. **(C)** Overlapping cellular processes and pleiotropic molecular mechanisms involving CDK5 across development and in the adult brain. aRG, apical radial glia; bRG, basal radial glia; CP, cortical plate; E, embryonic day; IN, interneuron; IPC, intermediate progenitor cell; N, excitatory neuron; pcw; post-conception week; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter.



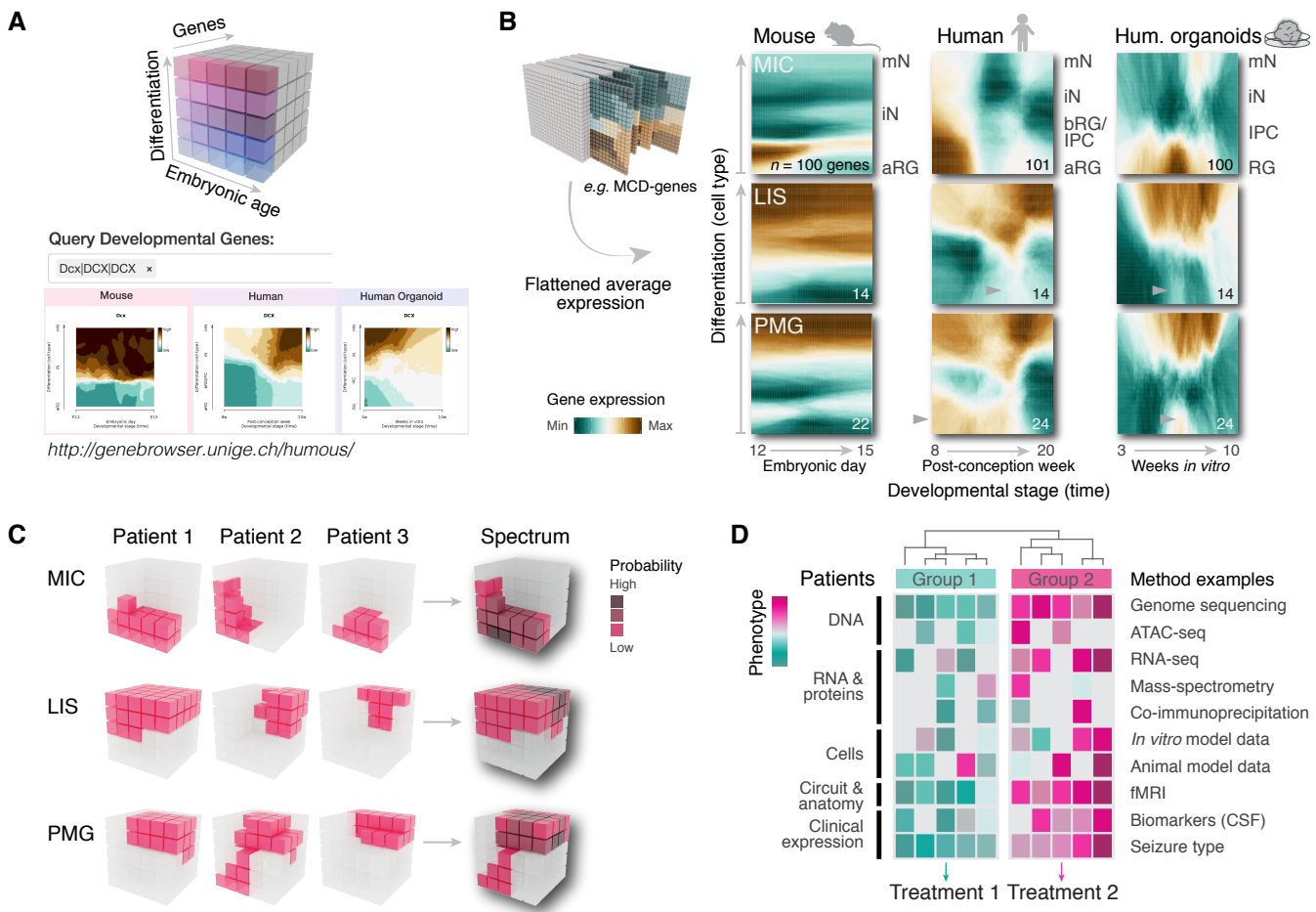
**Fig. 2: Cellular mechanisms at play in human cortical malformations.** Cells with abnormal developmental trajectories are in pink. Reduced progenitor proliferation (sometimes associated with abnormal neuron migration) leads to microcephaly. Abnormal neuron position can lead to agyria and lissencephaly (reduced migration and/or progenitor misposition), polymicrogyria (increased migration and/or excess of basal radial glia), cobblestone lissencephaly (excessive migration), periventricular heterotopia (neurons stay close to the ventricle) or subcortical band heterotopia (neurons stay below the cortex). In focal cortical dysplasia, clonal mutations affect subpopulations of mature cells, which become dysmorphic (balloon cells, dysmorphic neurons). Genes mentioned in this review are listed (bold: genes with published human-derived brain organoid models, \*: gene studied in the ferret, green: genes coding for transcription factors). Pink arrowheads on MRIs highlight the malformations. MRI illustrations are from (15) and (2) for polymicrogyria and focal cortical dysplasia (Reprinted from *Semin Cell Dev Biol* vol 76, D. M. Romero, N. Bahi-Buisson & F. Francis, Genetics and mechanisms leading to human cortical malformations, pages 33-75, 2018, with permission from Elsevier; Definitions and classification of malformations of cortical development: practical guidelines; M. Severino et al., *Brain* 2020; awaa174, with permission from Oxford University Press).



**Fig. 3: From gene mutations to diseases: levels of complexity.** (A) Levels of organization during corticogenesis, from DNA (*i.e.* genes, represented by letters) to RNA and proteins (*i.e.* gene expression), to cells, circuit and anatomy, and phenotype. Each circle represents a given feature of that level (*e.g.* a gene, a protein, a circuit). Interactions within levels are linked through complex relationships (dashed lines) to states at other levels (black lines). (B-G) Examples of abnormal feature relationships across levels in disease (highlighted in pink). In B-D, linear (monogenic), convergent (polygenic) and divergent relationships between genetic defects and phenotype(s) are illustrated. (E) Hidden complexity: note that from a gene-phenotype point of view, this relationship appears linear. (F) Hidden hub: different genes may lead to distinct phenotypes *via* hidden feature hubs influencing several downstream pathways. (G) Incomplete penetrance: not all genetic features are expressed at other levels. In A, source images for human brain section and phenotype are from <https://msu.edu/~brains/brains/> and ©biorender.com, respectively.



**Fig. 4: Spatiotemporal and cell type regulations of gene expression and malformations. (A)** Alternative splicing of *FLNA* gene regulates its role in proliferation *versus* neuron differentiation of cortical progenitors (top). Des-inhibition of the *FLNA* poison exon leads to precocious neuron differentiation and periventricular heterotopia in humans (bottom, pink arrowheads). **(B)** *DCX* mutation in human is associated with subcortical band heterotopia (pink arrowheads). In mice, constitutive *Dcx* loss of function does not cause major cortical defects, while acute uni/bilateral loss of function induces large subcortical heterotopia. Heterotopia are highlighted in pink. KO, knock-out. **(C)** The numbers of publications containing the indicated combinations of keywords (cell types and neurodevelopmental diseases) were identified with the EasyPubMed R package as described in Fig. 1. **(D)** Basic connectivity rules in the mouse somatosensory cortex. This connectivity appears largely conserved when neurons are mispositioned, like in the “scrambled” cortex of *Reeler* mice or in case of heterotopia. ASD, autism spectrum disorder; FCD, focal cortical dysplasia; HET, heterotopia; L, layer; LIS, lissencephaly; MIC, microcephaly; PMG, polymicrogyria; SZ., schizophrenia. In B, source image for mouse is from ©biorender.com.



**Fig. 5: Multimodal assessment of neurodevelopmental disorders. (A)** Identification of developmental gene dynamics in specific cell types through single-cell RNA sequencing (top). Illustration from the online resource <http://genebrowser.unige.ch/humous/> comparing data from mouse, human embryos, and human derived- brain organoids (bottom). **(B)** Average expression of genes associated with microcephaly (MIC), lissencephaly (LIS) and polymicrogyria (PMG) in mouse, human embryos, and human derived- brain organoids. Arrowheads show human-specific patterns. **(C)** Schematic representation of the probabilistic distribution of affected processes in sample disorders. Pink cubes define abnormal states. **(D)** Hypothetical patient classification through integration of multiple datasets. Here patients were clustered into 2 groups based on the integration of several features, from genes to clinical expression. ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing) allows to identify chromatin accessible regions. Mass-spectrometry and co-immunoprecipitation allow to identified proteins based on their molecular weight and interaction(s) between proteins, respectively. *In vitro* and animal model data bring complementary information about the cellular effect(s) of a gene mutation. aRG, apical radial glia; bRG, basal radial glia; CSF, cerebrospinal fluid; fMRI, functional magnetic resonance imaging; iN, immature neurons; IPC, intermediate progenitor cells; MCD, malformations of cortical development; mN mature neurons. In B, data from mouse embryos are from (94); data from human embryos and human-derived organoids are from (95, 96). The 2-dimensional landscapes of gene expression across time and differentiation were performed as described in (94). In B, source images for mouse and human are from ©biorender.com.