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CASE REPORT

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Neocortical neuronal arrangement in Miller Dieker syndrome

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Abstract Miller Dieker syndrome (MDS, type I lissencephaly) is a neuronal migration disorder, which is caused by deletions along the short arm of chromosome 17 (17p13.3). Recent studies would suggest that the cortical lamination in MDS is inverted, based on morphological criteria. The present neuropathological study examines the cerebral cortex from a 33-week old fetus with MDS using both neuronal and laminar-specific markers. These expression studies demonstrate a relatively preserved cortex and cortical lamination, overlying a layer of immature neurons in MDS brain. The findings are consistent with both a migratory and proliferative defect, giving rise to lissencephaly. Moreover, characterization of such rare human malformations of cortical development by immunohistochemical techniques will provide a greater understanding of the underlying mechanisms.

Keywords Miller Dieker syndrome · Lissencephaly · Neuronal migration · Neuronal proliferation · CNS · Cortical development · Cortical lamination · Neuropathology

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Introduction

Type I lissencephaly (meaning smooth brain, OMIM 607432) is a malformation of cortical development, characterized by the absence of sulci and gyri (brain convolutions) [4, 16]. The disorder can be caused by mutations in the *LIS1* gene on the short arm of chromosome 17 (17p13.3). In cases where there is a deletion in the 17p13.3 region [so termed Miller Dieker syndrome (MDS)], the lissencephaly is often more severe and associated with facial abnormalities. Previous neuropathological studies have demonstrated a ''four-layered'' cerebral cortex in type I lissencephaly/MDS, with the external cellular layer comprised of presumptive deep layer V/VI pyramidal neurons [8]. These findings have raised the suggestion that the cortex in type I lissencephaly is inverted, with earlier born neurons forming the more superficial layers and later born neurons forming the deeper layers [18]. No prior studies have used immunohistochemical markers to characterize the layering in MDS cortex. We describe expression patterns of several neuronal and laminar markers in the MDS cerebral cortex, which suggest a relatively preserved cortex overlying a large deep layer of less mature neurons that have failed to migrate into the cortex.

Case report

Diagnosis of classical lissencephaly prenatally was made by ultrasound and a deletion in the MDS region was confirmed by fluorescent in situ hybridization on chro-

mosomal testing. On gross examination, the 33 weeks GA male fetus had a dysmorphic facies (prominent forehead, bitemporal hollowing, short nose with upturned nares, prominent upper lip with downturned vermilion border and small jaw), characteristic of MDS. Clinically, the male fetus was born through uninduced labor at 33 weeks GA (32 weeks GA by crown rump 29.2 cm), but expired within minutes after birth from respiratory failure. No maceration or other CNS insult was associated with the birth. Additional body measurements were consistent with 32–33 weeks GA (weight 1,460 grams, crown-heel 41.9 cm, head circumference 26.7 cm, and foot length 6.2 cm). The viscera were largely unremarkable except for a distally dilated large bowel with ganglion cells.

Materials and methods

Immunohistochemistry was performed on paraffin embedded sections from age-matched normal human cortex and cortex derived from the 33-week age stational fetus with MDS. The aged-matched control was a normal fetus that was spontaneously aborted following a motor vehicle accident. Antibody staining assessed for proliferation (Ki-67, 1:100, DAKO), migration (FOXP1, A. Banham, using previously described method [1], neuronal differentiation (TBR-1, 1:200 dilution, courtesy Dr. Hevner; NSE, Zymed, stock; Neu-N, Chemicon, 1:1500) and the neuroepithelial lining (α-catenin and β-catenin, Becton Dickenson, 1:200 dilution). Cell death detection by TUNEL staining follows methods outlined by the manufacturer (In Situ Cell Death Detection kit, TMR red, Roche Diagnostics).

Neuropathological findings

The brain was examined within 12 h post-mortem and weighed 147 g (NL 196 g). Coronal sections were notable for the loss of sulci and gyri (cortical folds) on the surface of the brain, and ectopic large olivary heterotopia in the brain stem (Fig. 1)—both typical features for this disorder [2, 4]. Microscopic examination of brain sections stained with hematoxylin and eosin demonstrated the characteristic "four-layered" cortex seen in this disorder; these lamina corresponded to (1) a cell sparse molecular layer, (2) a highly cellular layer, (3) a cell sparse layer, and (4) a large cell dense band, filled with heterotopic neurons.

Immunohistology using several neuronal and layer specific markers was performed to examine neuronal cell morphologies and cortical lamination. Staining with the antisera to the laminar-specific transcription factor FOXP1 demonstrated that the deep pyramidal neurons in layer V/VI of the normal cortex were found in layer 3 of the MDS cortex (Fig. 2). Furthermore, staining with antisera to neuron-specific enolase (NSE) showed that the pyramidal neurons similar to those in layers III and V/VI of normal cortex can be appreciated in layers 2a and 3 of MDS cortex (Fig. 3). The preserved layering of these two pyramidal neuron populations indicates that surprisingly, there may not be a gross laminar inversion in MDS cortex. In contrast to mouse cortex where immunostaining for the putative transcription factor TBR-1 labels the deep layer neurons, in human cortex no preferential laminar staining of neurons was observed. However, the various neuronal morphologies observed with TBR-1 staining suggested that similar neuronal morphologies, observed in the first three layers of the

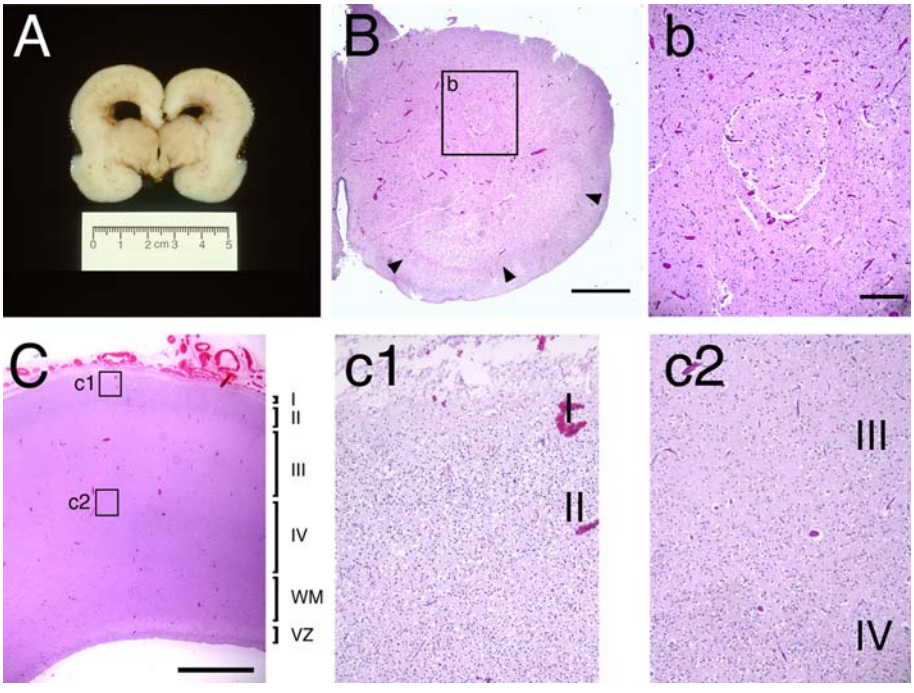

Fig. 1 Histopathology in human MDS. **a** Gross specimen of the brain from a 33-week old fetus with confirmed MDS. Brain is lissencephalic (lacking in sulcal and gyral folds) with a thickened cortex. **b** Periodic acid Schiff stain of the midbrain. MDS is characterized by heterotopic nodules in the brainstem and dysplastic inferior olivary nucleus (*arrowheads*). **b** Inset in (**b**) shows abnormal heterotopia suggestive of a migrational disorder. **c** The cortex is abnormally thickened (4 cm as opposed to 1 cm). (c1,c2) Higher magnification photomicrographs of (**c**) demonstrate the characteristic four layers in MDS as opposed to the six distinct layers seen in normal cortex. *Scales bars* in b, c = 1 mm and b, c1, c2 = 100 microns

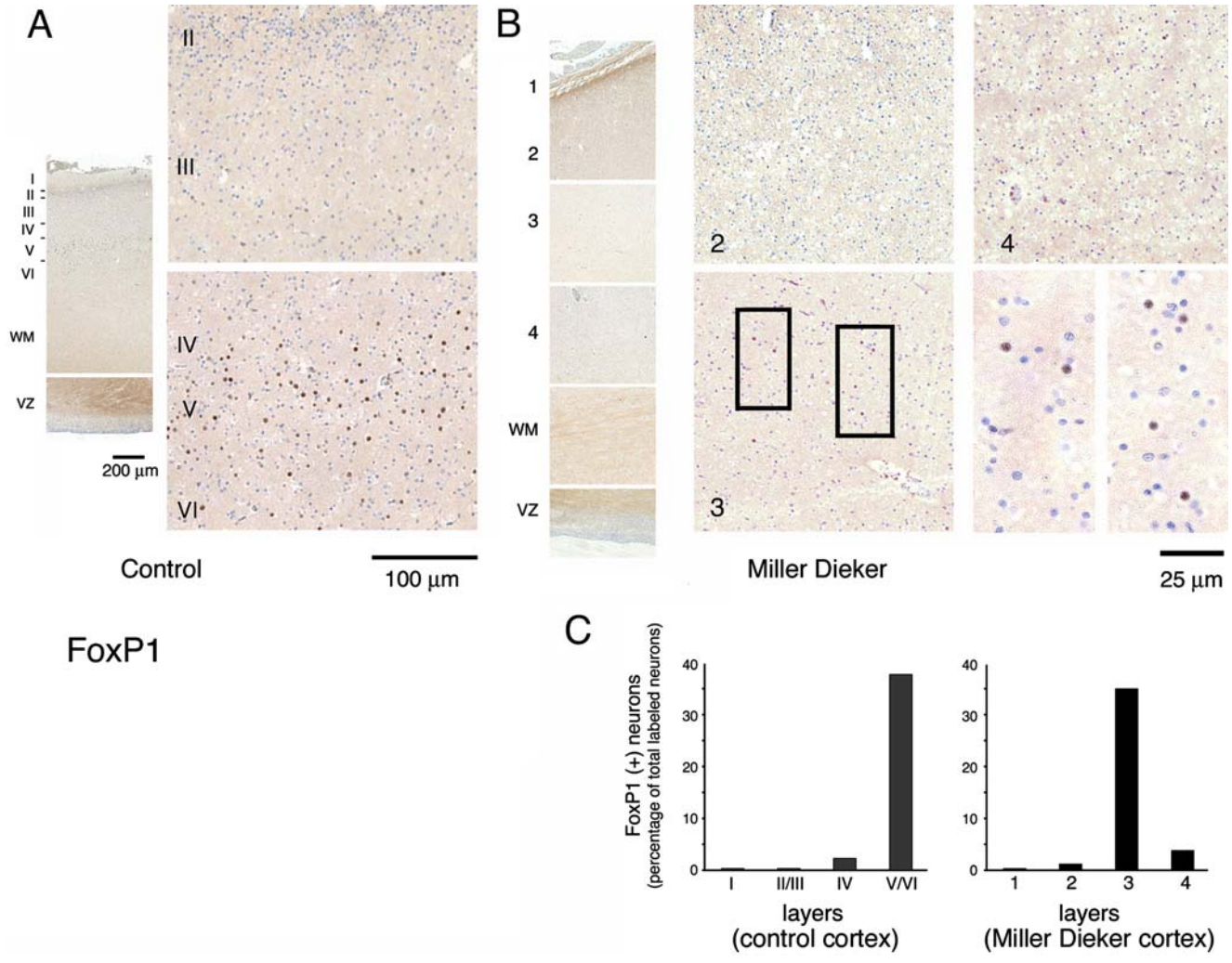

Fig. 2 Deep pyramidal neurons in layer V/VI of the 33-week gestational age normal human frontal cortex correspond to deep pyramidal neurons in layer 3 of MDS cortex by layer-specific FOXP1 staining. **a** The layer-specific transcription factor FOXP1 localizes to laminar V/VI in the control cerebral cortex. **b** FOXP1 staining in layer 3 but not layer 2a of MDS cortex suggests that some aspects of the cortical lamination are preserved. **c** Quantification of the laminar location of FOXP1+ cells is graphically illustrated. These results suggest that pyramidal neurons in layers V/VI of normal cortex correspond appropriately with pyramidal neurons in layer 3 of MDS cortex, indicating that there is no laminar inversion in MDS cortex. *Scale bars* are as indicated

MDS cortex, again corresponded appropriately in spatial position with the neuronal morphologies, observed in the six layers of normal human cortex (Fig. 4). For example, the hypocellular molecular layer I in normal cortex looks similar to the hypocellular layer I of MDS cortex. In general, the granule and pyramidal neurons in layers II–IV bear resemblance to layer 2a, b of MDS cortex. The deep pyramidal neurons in V/VI of normal cortex can be compared to layer 3 of MDS cortex. MDS cortex, however, also appears to have an underlying band of immature neurons with various morphologies. Neurons in this dense MDS hypercellular layer 4 appear small and immature by TBR-1, and virtually none are stained by the mature neuronal marker NeuN (Fig. 5). Finally, immature neurons in this layer 4 lie beneath the FOXP1 neurons, which characteristically stain the deepest layers of normal cortex.

Immunohistochemistry using several proliferative and cell death specific markers was performed to examine the cerebral wall. As previously observed, gross autopsy examinations generally show that the MDS brain is significantly smaller relative to the other organ systems of the body and weighs less than a normal brain for a 33-week old fetus (Fig. 6a). Staining with the neuroepithelial markers α- and β-catenin demonstrates a dramatic disruption of the neuroepithelium along the lateral ventricles (arrows, the region responsible for the expansion of the neural progenitors giving rise to post-mitotic cortical neurons (Fig. 6b). The few remaining proliferative cells at this fetal age are stained by the cell proliferative marker Ki-67 and appear inappropriately localized to this abnormal ventricular lining (Fig. 6c), consistent with some disturbance in neural precursor proliferation. Finally, TUNEL staining for programmed

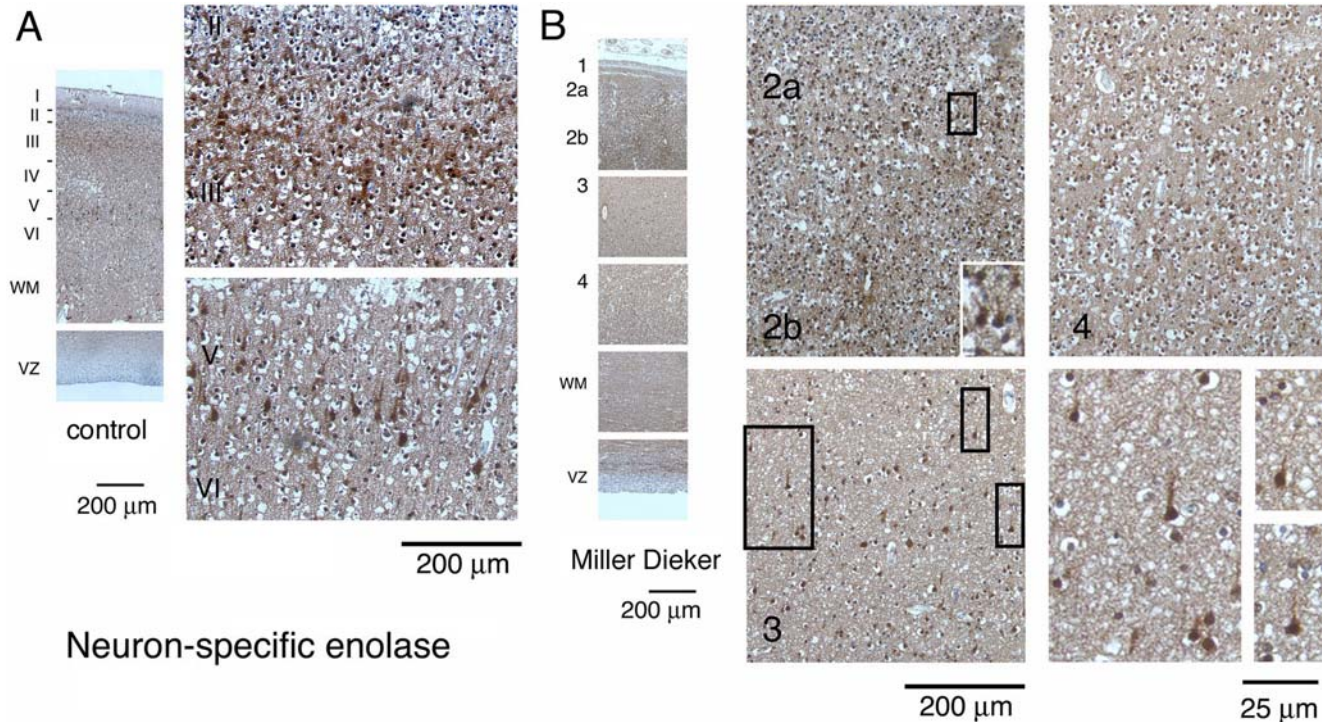

Fig. 3 Pyramidal neurons in layers III and V/VI of the 33-week gestational age normal human frontal cortex correspond appropriately to pyramidal neurons in layers 2a and 3 of age-matched human MDS frontal cortex. **a** Coronal sections demonstrate the cytoplasmic staining of the neuronal-specific marker NSE in the pyramidal neurons, residing in layers III and V/VI of normal cortex. **b** Coronal sections stained for NSE in MDS cortex similarly suggests that pyramidal neurons reside in layers 2a and 3. These results suggest that pyramidal neurons in layers III and V/VI of normal cortex correspond, respectively with pyramidal neurons in layers 2a and 3 of MDS cortex. *Scale bar* sizes are as indicated

cell death shows that the MDS cortex has an increased number of apoptotic cells (Fig. 6d).

Cytogenetic and molecular investigations

Karyotyping performed on amniotic fluid cells in cultures showed a partial trisomy of 1p and partial monosomy of 17p. The mother carried a balanced *t* (1;17). In situ hybridization using ONCOR (6C266) probe detected a 17p13.3 deletion. Microsatellite analysis of the region for loss of heterozygosity showed a typical 3.5 Mb deletion region between markers D17s1840 and D17s1876, which includes both the *YWHAE* and *LIS1* genes.

Discussion

The current study using layer and neuronal markers suggests that the cortical lamination in MDS brain may be relatively preserved with the exception of the subcortical arrest of some small neurons and with the layers being more disorganized and expanded across the width of cortex. While some prior histological studies have suggested that the cortical ribbon displays an inverted organization [18], these observations were based solely on morphological criteria. Moreover, the current findings of a non-inverted lamination are consistent with the heterozygous *lis-1* mice, which are reported to demonstrate only a mild neuronal migration defect [9, 10]. Finally, the presumptive function of LIS1 in binding to dynein would suggest some impairment in cell motility or perhaps cell division given its direct interaction with the cytoskeleton, and not a problem in cell arrest (as seen in the reeler mutation) [5, 12].

The relative preservation of the cortex overlying an abnormal band of immature, heterotopic neurons may arise from impairments in several developmental processes. Mutations in lis1 have clearly been shown to result in slow or delayed neuronal migration from the ventricular zone in mice [9, 10]. The loss of interneurons in the MDS human and lis1 mouse cortex are also consistent with the inability of inhibitory neurons to migrate long distances from the ganglionic eminence into the cerebral cortex [15] However, recent studies suggest that Lis1 interacts with several centrosomal proteins (Nde1 and Nudel) [6, 7], raising the possibility that altered proliferation through prolongation of the cell cycle could lead to delayed exit of neurons from the VZ and thereby contribute to an underlying band of more immature appearing neurons. Compound null/hypomorphic Lis1 mice (expressing only 35% of the Lis1 protein) also show impairments in neuroblast generation [9]. Furthermore, human MDS neural progenitors, isolated and expanded from postmortem human

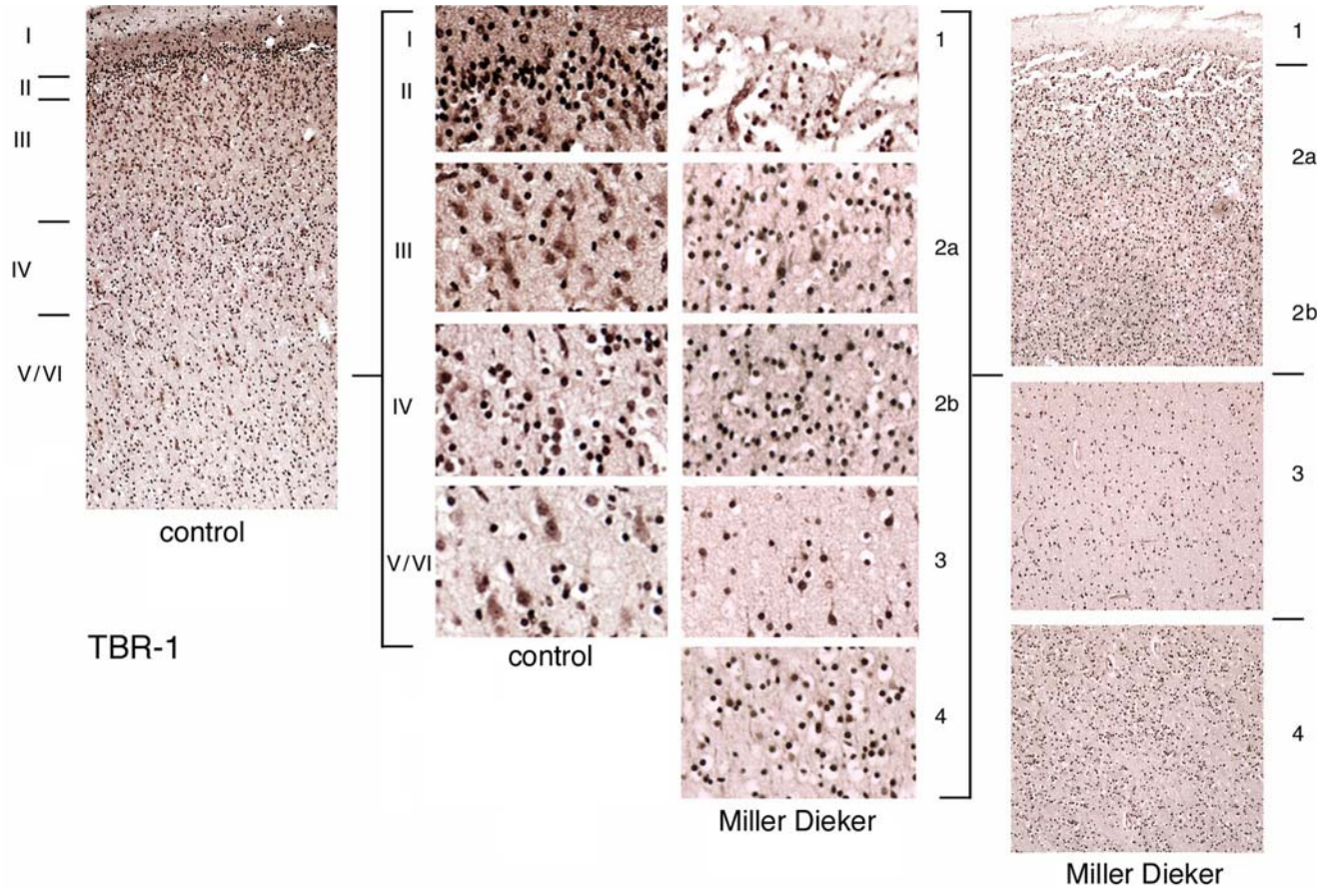

Fig. 4 Shared neuronal morphologies in the different cortical layers of 33-week gestational age normal and MDS human frontal cortex. Immunostaining with an antisera against the neuronal transcription factor TBR-1 in coronal sections of normal human frontal cortex (*far left*) demonstrates the characteristic six layers of neocortex, (I) the cell sparse molecular layer, (II) the external granule cell layer composed of small pyramidal and stellate neurons, (III) the external pyramidal layer composed of medium-sized pyramidal neurons, (IV) internal granule layer composed of small stellate neurons, (V/VI) the internal pyramidal layer composed of medium to large pyramidal neurons and neurons of various morphologies. Higher magnification photomicrographs directly to the right demonstrate the various neuronal morphologies for the respective layers. TBR-1 immunostaining in the MDS human frontal cortex (*far right*) demonstrates the characteristic four layers, (1) the cell sparse molecular layer, (2) the hypercellular external cell layer composed of pyramidal neurons (2a), and stellate neurons (2b), (3) the hypocellular layer composed of large pyramidal neurons and (4) the deeper, large hypercellular layer composed of presumably more immature neurons of various morphologies. Higher magnification photomicrographs directly to the left demonstrate the various neuronal types. In many respects, there is an appropriate correspondence of cell types between the normal and MDS cortex for the corresponding layers

brain, appear to have impairments in proliferation and undergo increased cell death (Sheen and Walsh, personal observations). The current observations of a reduction in brain size and weight, disruption at sites of neural proliferation and the increased programmed cell death are consistent with impairments in proliferation. Finally, a delayed exit from the VZ due to a mitotic delay would, in part, explain the observation of immature (later born) neurons in the deep layer 4 of the lissencephalic cortex. Alternatively, these cells may not undergo appropriate differentiation although many of the neurons residing in the overlying cortex and harboring the *LIS1* deletion do morphologically resemble neurons appropriate for the respective layers.

Lissencephaly may arise from processes that disrupt intracortical lamination rather than merely from impairments in neuronal migration. The identification of causative genes, such as *LIS1* and *DCX*, suggest a problem in cell motility given their inferred structure and presumptive interaction with the cytoskeleton [17]. However, the induction of cell death can lead to lissencephaly in ferrets [11, 14], proliferative and cell death defects are appreciated in the MDS neural precursors (Sheen, personal observations), and microcephaly has been associated with other forms of lissencephaly [13]. Moreover, cell arrest defects in lissencephaly with cerebellar hypoplasia (*RELN*) [17] can also contribute to the loss of cortical folds. Finally, in cobblestone lissencephaly, neurons migrate beyond the surface of the brain, resulting in a disorganized and thinner inner cortex [17]. Overall, the common endpoint resulting from each of these genetic defects is a disruption in

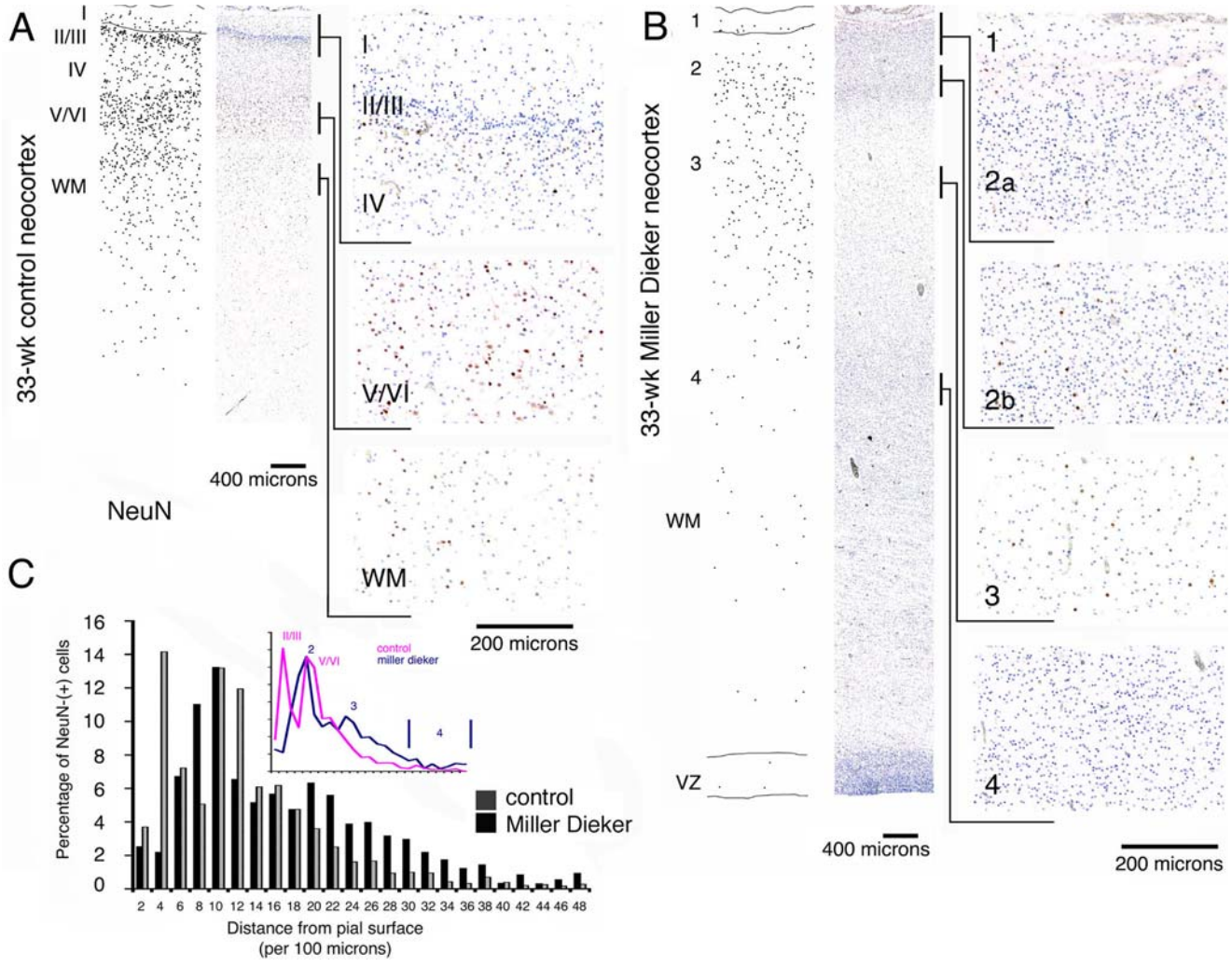

Fig. 5 Disorganization and expanded width of the different layers in MDS cortex overlying a band of immature neurons. **a** Camera lucida drawing (*left*) of a representative coronal section stained for the neuronal marker NeuN (*right*) from a normal 33 gestational week old cortex. The majority of neurons are positioned characteristically in two bands, corresponding to layers II/III and V/VI of the neocortex. **b** Camera lucida drawing (*left*) of a representative coronal section stained for the neuronal marker NeuN (*right*) from a 33 gestational week old MDS cortex similarly demonstrates two bands (layers 2 and 3). Additionally, a large hypercellular band (layer 4) with weak NeuN staining suggestive of earlier born neurons persists beneath this cortex. **c** Quantitative histogram of the relative distribution and distance of NeuN labeled neurons. The neuronal distribution of the MDS cortex parallels that seen in the normal cortex, albeit the layers are expanded, and less uniform. This cortex overlies a large, hypercellular layer of immature neurons. *Scale bar* sizes are as indicated

cortical lamination, either from neurons delayed in reaching their destination, too few neurons reaching their destination, neurons that do not know when they have reached their destination or neurons that migrate beyond their intended destination. Furthermore, the temporal sequence of sulci and gyri development argues that gyrification is an intracortical process, largely because cortical proliferation and migration are complete before the onset of gyrification in humans and the severing of cortical projections does not abrogate sulci and gyri formation [3]. These observations collectively suggest that differential growth in upper and lower cortical layers may be a primary force contributing to the formation of cortical folds.

The increasing availability of selective neuronal and laminar specific markers now provides the opportunity to study different aspects of development from a single static time-point in development. Further characterization of these rare human cases of lissencephaly by such immunohistochemical techniques will provide a greater understanding of the mechanisms leading to these malformations of cortical development.

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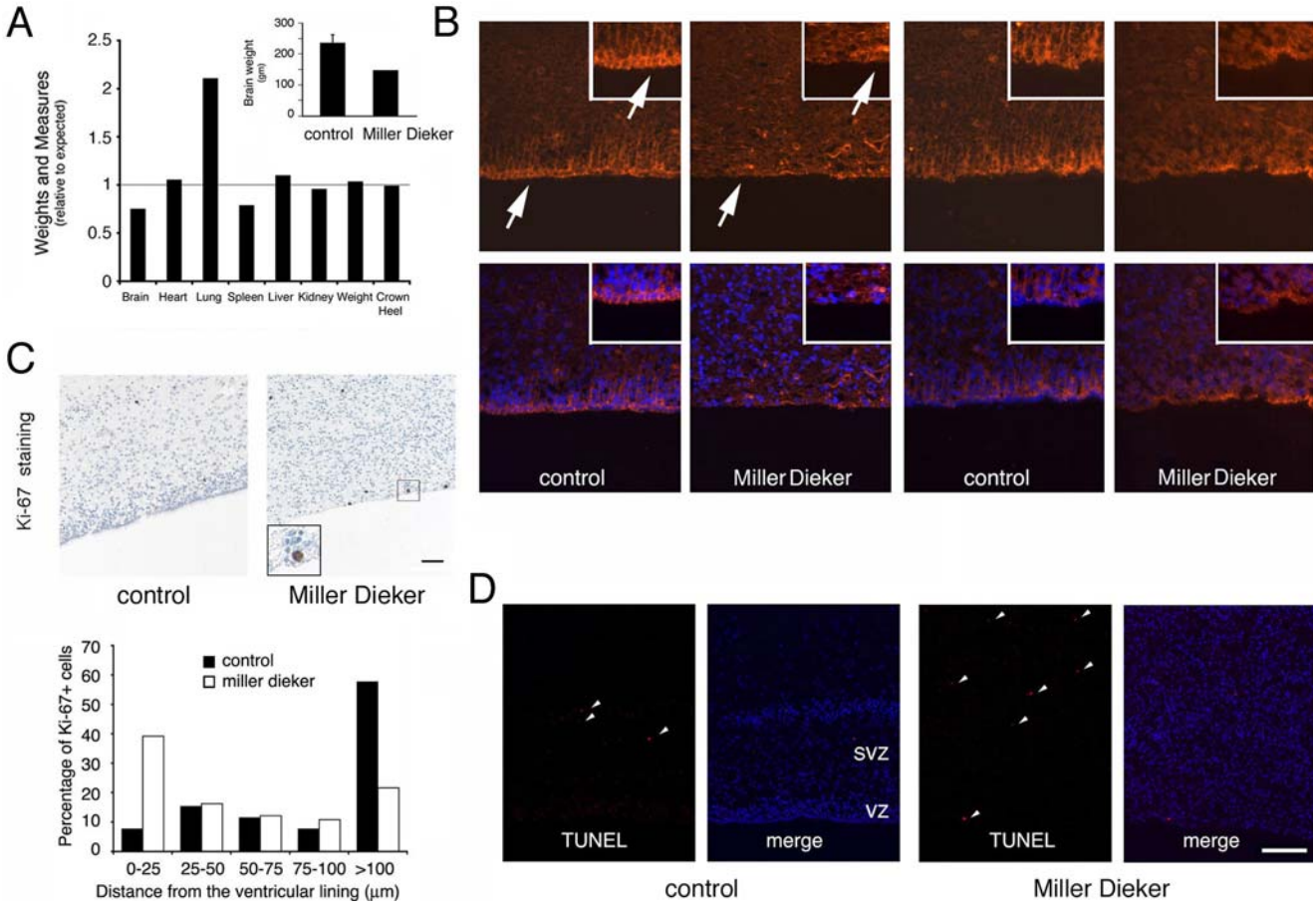

Fig. 6 Impaired proliferation, increased cell death, and disruption of the neuroependyma along the ventricular zone (*VZ*) of MDS brain. **a** The MDS brain is smaller relative to other organ systems from the same affected patient. The MDS brain also weighs less relative to age-matched control brain. **b** The proliferative region along the neuroependyma of the lateral ventricles (*arrows*) is disrupted in MDS brain. Rhodamine and merged (rhodamine and DAPI) fluorescence photomicrographs show loss of the highly structured expression of the neuroepithelial markers α- and β-catenin within MDS cortex as compared to control. **c** Light field photomicrograph of the ventricular zone (*VZ*) and subventricular zone (*SVZ*) shows proliferative Ki-67+ cells, abnormally restricted along the neuroependyma, further suggesting a disruption in cell proliferation. The higher magnification boxed inset shows a Ki-67+ cell, labeled by DAB and counterstained with Giemsa. (D) Fluorescence photomicrographs of the VZ and SVZ of a 33 gestational week human with MDS are suggestive of increased cell death by TUNEL stain (*arrowheads*, rhodamine fluorescence) compared to control. *Scale bars* in $D = 200$ microns, $E = 100$ microns

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