ARTICLE

Congenital Microcephaly

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The underlying etiologies of genetic congenital microcephaly are complex and multifactorial. Recently, with the exponential growth in the identification and characterization of novel genetic causes of congenital microcephaly, there has been a consolidation and emergence of certain themes concerning underlying pathomechanisms. These include abnormal mitotic microtubule spindle structure, numerical and structural abnormalities of the centrosome, altered cilia function, impaired DNA repair, DNA Damage Response signaling and DNA replication, along with attenuated cell cycle checkpoint proficiency. Many of these processes are highly interconnected. Interestingly, a defect in a gene whose encoded protein has a canonical function in one of these processes can often have multiple impacts at the cellular level involving several of these pathways. Here, we overview the key pathomechanistic themes underlying profound congenital microcephaly, and emphasize their interconnected nature. © 2014 Wiley Periodicals, Inc.

KEY WORDS: cell division; mitosis; DNA replication; cilia

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INTRODUCTION

Congenital microcephaly, an occipital-frontal circumference of equal to or less than 2–3 standard deviations below the age-related population mean, denotes a fundamental impairment in normal brain development [Woods and Parker, 2013]. Depending on the underlying cause, congenital microcephaly can be associated with structural brain malformations [e.g., gyrification issues, agenesis of corpus callosum, pituitary abnormalities] or secondary consequences such as craniosynostosis [Verloes et al., 2013]. Congenital microcephaly can have an environmental or genetic etiology [Gilmore and Walsh, 2013]. Cerebral cortical neurons must have developed by mid-gestation although glial cell division and consequent brain volume enlargement does continue after birth [Spalding et al., 2005]. Impaired neurogenesis is therefore most obviously reflected clinically as congenital microcephaly.

Fundamentally, neurogenesis incorporates several stages that are very susceptible to problems in the efficient and effective execution of genome maintenance, DNA replication and ultimately cell division. The developing human neuroepithelium must undergo a rapid expansion in stem cell numbers to fuel its own symmetric expansion [Rakic, 1995]. This is essential to generate enough capacity to instigate and maintain asymmetric division for neuronal differentiation, enabling the formation of the various cortical layers. Furthermore, differentiating and developing neurons must migrate to their defined locations to construct the complex architecture and laminar layered structure of the cortex [Tan and Shi, 2013; Wu et al., 2014] (Fig. 1).

What spectrum of physiological deficits underlies congenital microcephaly? Defects resulting in elevated levels of apoptosis can deplete neuroprogenitor stem and differentiating cells. Defects impacting upon efficient DNA replication can limit the capacity of the neuroepithelium to expand under its strict temporal constraints. Defects in the mitotic apparatus (e.g., microtubule spindles, centrosomes, centrioles) can lead to impaired symmetric-asymmetric

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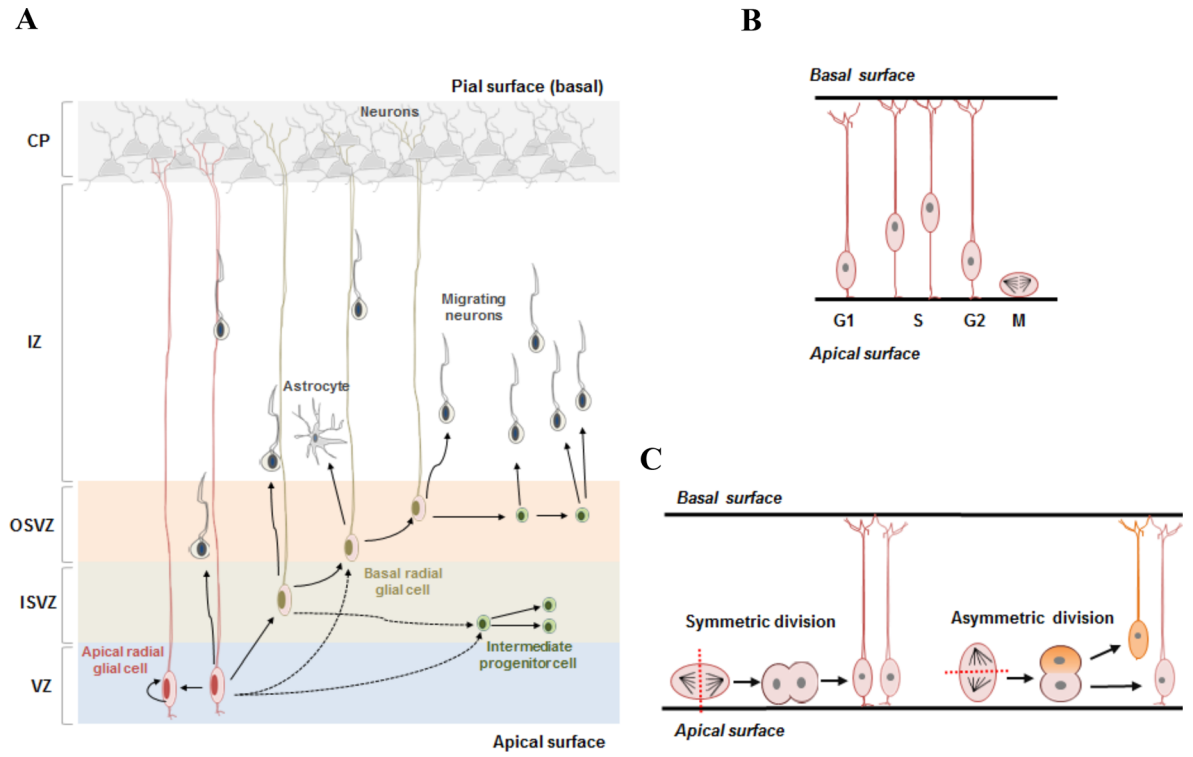

Figure 1. Cell division and differentiation in the developing cerebral cortex. (**A**). Neural plate originating cells initially undergo a phase of symmetric self-renewal and lateral expansion. Then, as development continues they asymmetrically divide to give rise to glial cells and neurons in the cerebral cortex, which ultimately structures into multiple discrete layers following development. Radial glial cells are key neuroprogenitors whose cell body lies in the ventricular zone (VZ) and whose radial fibers span the width of the cerebral cortex. These can differentiate into neurons, as well as intermediate progenitors that migrate to the subventricular zone (SVZ) and can further generate more progenitor cells and neurons by repeated asymmetric cell division. Finally, radial glial cells also can give rise to astrocytes and oligodendrocytes at later stages. Additionally, the radial glial fibers function as scaffolding for migrating cells to move outwards and away from the VZ to their appropriate destination regions. Ultimately the migrating neurons become the pyramidal cells of the cerebral cortex. CP: cortical plate. IZ: intermediate zone. VZ: ventricular zone. OSVZ: outer-subventricular zone. ISVZ, inner-subventricular zone. IZ: intermediate zone. (**B**) Neuroepithelial cell division within the ventricular zone (VZ) is accompanied by nuclear migration between the apical and basal surfaces of the developing cerebral cortex. In the G_1 phase of the cell cycle, the cell nucleus ascends from the apical surface towards the basal end of the ventricular zone, where it remains during S-phase. During G_2, the nucleus then returns to the apical surface where mitosis (M) takes place. (**C**) Proliferative *versus* neurogenic (differentiating) cell division is dictated by the cleavage plane orientation (dotted red line) of dividing neuroepithelial (NE) cells. Orientation of the mitotic spindle is parallel to the apical surface during symmetric cell division, with the resulting cleavage plan intersecting the apical plasma membrane (PM). A deviation in the orientation of the mitotic spindle results in an asymmetric cell division, with only one of the daughter cells inheriting the apical PM region (pink cell). The correct orientation of the mitotic spindle is therefore crucial for neurogenesis. The number of rounds of symmetric proliferative divisions undergone by NE cells determines the ultimate number of neurons. As neurogenesis progresses, increased asymmetric divisions means that only one daughter cell inherits the apical PM and remains a NE cell, while the other becomes either a basal progenitor, a radial-glial cell or a neuron (depicted as the orange cell). Additionally, cell cycle length plays a role in proliferative *versus* differentiating cell divisions. Longer cell cycles have been associated with differentiating divisions, while shortening of G1 phase for example has been linked to an increase in proliferative divisions.

division, inappropriate cell cycle arrest and/or elevated apoptosis. Mutations in genes encoding key players in each of these biological processes have been described in patients exhibiting profound congenital microcephaly (Tables I–III). This can present as the most marked clinical feature; primary microcephaly (PM), or in association with pronounced growth restriction; as in the growing family of genetically identifiable microcephalic primordial dwarfisms (MPDs), such as Seckel syndrome, microcephalic primordial dwarfism types I and II and Meier–Gorlin syndrome. Herein, we overview the genetic defects associated with severe congenital microcephaly and discuss how they contribute to the general phenomena of limiting cell division capacity and elevating levels of stem and/or neuroprogenitor cell death as mechanisms underlying impaired neurogenesis.

MITOSIS AND MICROCEPHALY

To date, the majority of the genetic defects identified in PM and MPDs involve genes encoding proteins that play fundamental roles in various processes that collectively enable cells to

TABLE I. Centrosome and Spindle Microtubule Defects Underlying Severe Congenital Microcephaly

Gene	Protein	OMIM	Clinical presentation	Localization and function
MCPH1	Microcephalin	251200	PM. Cells exhibit premature chromosome condensation	Centrosome-role in DNA repair and G2-M dynamics
WDR62 (MCPH2)	WD-repeat containing protein 62	604317	PM	Mitotic spindle pole formation-scaffold for JNK pathway
CDK5RAP2/ CEP215 (MCPH3)	Cyclin dependent kinase 5 regulatory subunit-associated protein 2	604804	PM	Centrosome, spindle and microtubule organizing function.
CASC5 (MCPH4)	Cancer susceptibility candidate 5	604321	PM	Kinetochore [KNM] component-spindle-assembly checkpoint
ASPM (MCPH5)	Abnormal spindle-like, microcephaly associated	608716	PM	Microtubule associated protein-spindle organization and orientation
CENPJ/CPAP (MCPH6)	Centromeric protein J	608393 613676	PM-MPD-Seckel syndrome	Centriole biogenesis-cilia formation
STIL (MCPH7)	SCL/TAL1 interrupting locus	612703	PM	Centrosome-centriole biogenegis
CEP135 (MCPH8)	Centrosomal protein 135kDa	614673	PM	Centrosome-centriole biogenegis
CEP152 (MCPH9)	Centrosomal protein of 152 kDa	614852 613823	PM-MPD-Seckel syndrome	Centrosome-centriole biogenesis and genome stability
CEP63	Centrosomal protein of 63 kDa	614728	Microcephaly with growth retardation-Seckel syndrome [mild]	Centrosome-centriole biogenesis
NDE1	Nuclear distribution protein nudE homolog 1	614019	Microlissencephaly microhydraencephaly	Centrosome-mitotic spindle
NIN	Ninein	614851	MPD-Seckel syndrome	Centrosome function-microtubule organization
PCNT	Pericentrin	210720	Microcephalic Osteodysplastic Primordial Dwarfism (MOPD) II	Component of pericentriolar material-scaffold for signaling molecules?
BUB1B	BUB1 Mitotic Checkpoint Serine/Threonine Kinase B1 (BUBR1)	257300	Growth retardation-microcephaly-cancer-Mosaic Variegated Aneuploidy [MVA]	Kinetochore kinase-role in spindle checkpoint
CENPE	Centromere protein E (CENP-E)	117143[a]	MPD	Microtubule capture and stabilization
KIF5C	Kinesin family member 5C	615282	Microcephaly and cortical malformations	Microtubule motor protein
KIF2A	Kinesin family member 2A	615411	Severe microcephaly-cortical malformations-early-onset epilepsy	Microtubule motor protein
KIF11	Kinesin family member 11	152950	Microcephaly with or without chorioretinopathy, lymphoedema and mental retardation	Microtubule motor protein-role in microtubule crosslinking and bipolar spindle formation
TUBG1	Tubulin-gamma complex associated protein 1	615412	complex cortical malformations-microcephaly (not all reported cases)	Structural component of the centrosome-role in microtubule nucleation
TUBB2B	Tubulin, beta 2B class IIb	610031	Microcephaly-spastic tetraparesis-severe intellectual disability-scoliosis	Microtubule component-binds to GTP
TUBA1A	Tubulin, alpha 1A	611603	Microcephaly-severe intellectual disability	Microtubule component
POC1A	Proteome of the centriole 1A	614783	MPD	Centriolar protein required for cilia formation

PM, primary microcephaly; MPD, microcephalic primordial dwarfism.
[a]Denotes the gene entry in OMIM.

TABLE II. Defects in the Origin Recognition Complex Core and Associated Components Underlying Meier–Gorlin Syndrome

Gene	Protein	OMIM	Clinical presentation	Localization & function
ORC1	Origin recognition complex subunit 1	224690	Meier–Gorlin syndrome	Component of the pre-replication complex-initiation of DNA replication
ORC4	Origin recognition complex subunit 4	613800	Meier–Gorlin syndrome	Component of the pre-replication complex-initiation of DNA replication
ORC6	Origin recognition complex subunit 6	613803	Meier–Gorlin syndrome	Component of the pre-replication complex-initiation of DNA replication, coordination of chromosome-replication and segregation
CDT1	Chromatin licensing and DNA replication factor 1	613804	Meier–Gorlin syndrome	Component of the pre-replication complex -origin licensing factor
CDC6	Cell division cycle 6	613805	Meier–Gorlin syndrome	Component of the pre-replication complex-loading MCM complex-role in cell cycle checkpoints

execute precise chromosomal segregation and mitotic division [Thornton and Woods, 2009; Mahmood et al., 2011; Verloes et al., 2013](Table I). The mitotic phase of the cell cycle involves an intricate and highly complex ballet of interactions and transactions occurring in an organized and inter-dependent fashion [Walczak et al., 2010]. These include chromosome condensation, bipolar mitotic microtubule spindle network formation and dissolution, along with chromosomal kinetochore-mediated nucleation and capture by spindle microtubules to instigate the amphitelic restraining of chromosomes for alignment at metaphase prior to segregation. The centrosome represents an important spindle microtubule organizing center [Bettencourt-Dias and Glover, 2007]. There is now an increasing list of examples of hypomorphic defects in genes encoding core components of the centrosome associated with PM and MPD (Table I and Figs. 2 and 3).

To date, the majority of the genetic defects identified in PM and MPDs involve genes encoding proteins that play fundamental roles in various processes that collectively enable cells to execute precise chromosomal segregation and mitotic division.

Very often, the precise roles of these proteins at centrosomes are rather opaque. For some, such as Pericentrin and γ-tubulin, these often have "structural" or "scaffold" functions attributed them [Zimmerman et al., 2004]. In most instances, descriptive impacts upon centriole and centrosome duplication, and consequently abnormalities in microtubule spindle organization, have been observed for defects in these proteins [Griffith et al., 2008; Rauch et al., 2008]. Furthermore, there is growing evidence that defects in some of these proteins have additional negative impacts upon the centrosomal localization of other centrosome proteins that have independently been identified as underlying defects of PM and/or MPD. Illustrative examples include the interplay between CEP152 and CEP63 or for CEP152 and CENPJ (CPAP) [Cizmecioglu et al., 2010; Sir et al., 2011]. These occurrences further highlight the interconnected and functional interplay between many of these proteins, explaining to some degree why defects herein present with a common clinical manifestation of congenital microcephaly.

Centrosomes and Spindles

The centrosome cycle is coordinated with the canonical cell cycle whereby the mother centrosome, inherited from the previous mitosis, must duplicate to generate a mother-daughter pair prior to G_2-phase where they then act as a microtubule organizing center at the

Currently, the working functional model to explain impaired neurogenesis in the context of a defect in a centrosomal protein is that these often result in supernumerary centrosomes, fragmented centrosomes and/or premature centriolar separation. Consequently, these can result in deficits in mitotic spindle microtubule nucleation when establishing a bipolar spindle.

TABLE III. Defects in DNA Damage Response and DNA Repair Proteins Associated With Congenital Microcephaly

Gene	Protein	OMIM	Clinical presentation	Localization & function
ATR	Ataxia telangiectasia and rad3-related	210600	MPD–Seckel syndrome	Protein kinase-apical DDR regulator-cell cycle checkpoint activation-role in DNA replication
ATRIP	ATR-interacting protein	606605[a]	MPD–Seckel syndrome	Essential ATR partner-binds to RPA-coated ssDNA-DDR-role in DNA replication
RBBP8/ CTIP	Retinoblastoma-binding protein 8/CTBP-interacting protein (CtIP)	606744	MPD–Seckel syndrome	DNA DSB resection-role in ATR recruitment to DSBs-associates with BRCA1 in regulation of cell cycle checkpoints
NBS1/ NBN	Nijmegen breakage syndrome 1 /nibrin	251260	Nijmegen breakage syndrome (NBS): microcephaly-immunodeficiency-cancer predisposition	Component of M-R-N complex with central role in DNA DSB repair
RAD50	DNA repair protein RAD50	613078	NBS-like disorder: microcephaly-intelletual disability-"bird-like" face-short stature	Component of M-R-N complex with central role in DNA DSB repair
MRE11A	Double-strand break repair protein MRE11A	600814[a]	NBS-like severe microcephaly	Component of M-R-N complex with central role in DNA DSB repair
PNKP	Bifunctional polynucleotide phosphatase and kinase	613402	Microcephaly-seizures-developmental delay	Role in DNA repair pathways (NHEJ, BER)
CDK6	Cyclin-dependent kinase 6	603368[a]	PM	Control of cell cycle and differentiation-centrosomal association in mitosis
BRCA1	Breast cancer type 1 susceptibility protein	113705[a]	Microcephaly-short stature-developmental delay-cancer predisposition	DNA repair-cell cycle checkpoint control-maintenance of genomic stability (HRR)
BRCA2	Breast cancer type 2 susceptibility protein	600185[a]	MPD	DNA repair-cell cycle checkpoint control-maintenance of genomic stability (HRR)
LIG4	DNA ligase 4	606593	Ligase IV syndrome: microcephaly-dysmorphic facial features-growth retardation-skin anomalies-pancytopenia.	DNA DSB repair (NHEJ) and V(D)J recombination
NHEJ1	Non-homologous end-joining factor 1	611291	Growth retardation-microcephaly-immunodeficiency	DNA DSB repair (NHE)] and V(D)J recombination
CHLR1/ DDX11	DEAD/H (Asp-Glu-Ala-Asp/Hi) box helicase 11	601150[a]	Warsaw breakage syndrome [WABS]: microcephaly-pre- and postnatal growth retardation-abnormal skin pigmentation	DNA helicase-genome stability
PHC1 (MCPH11)	Polyhomeotic-like protein 1	615414	PM-short stature	Component of polycomb group (PcG) multiprotein PRC1-like complex/ repression of transcription/role in chromatin remodelling and histone modification
DNA2	DNA replication helicase 2	601810[a]	MPD	Helicase-DNA repair-genome stability
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2	600375[a]	Microcephaly-growth deficiency-facial nerve palsy-skeletal abnormalities	DNA DSB repair (HR)-genome stability
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	194363[a]	MPD	DNA DSB repair (NHEJ)-genome stability
NHEJ1	Nonhomologous end-joining factor 1. Also called XLF/ Cernnunos	611291	Severe combined immunodeficiency (SCID)-microcephaly-growth retardation-IR sensitivity	DNA DSB repair [NHEJ]-genome stability
RECQL3	Bloom syndrome helicase (BLM)	210900	Bloom syndrome-microcephlay-growth delay-immune deficiency-cancer	HRR pathway HJ resolving helicase

PM, primary microcephaly; IR, ionizing radiation.

[a]Denotes the gene entry in OMIM. MPD: microcephalic primordial dwarfism. HRR: homologous recombination repair. NHEJ: non-homologous DNA end joining. M-R-N: MRE11-RAD50-NBS1 complex.

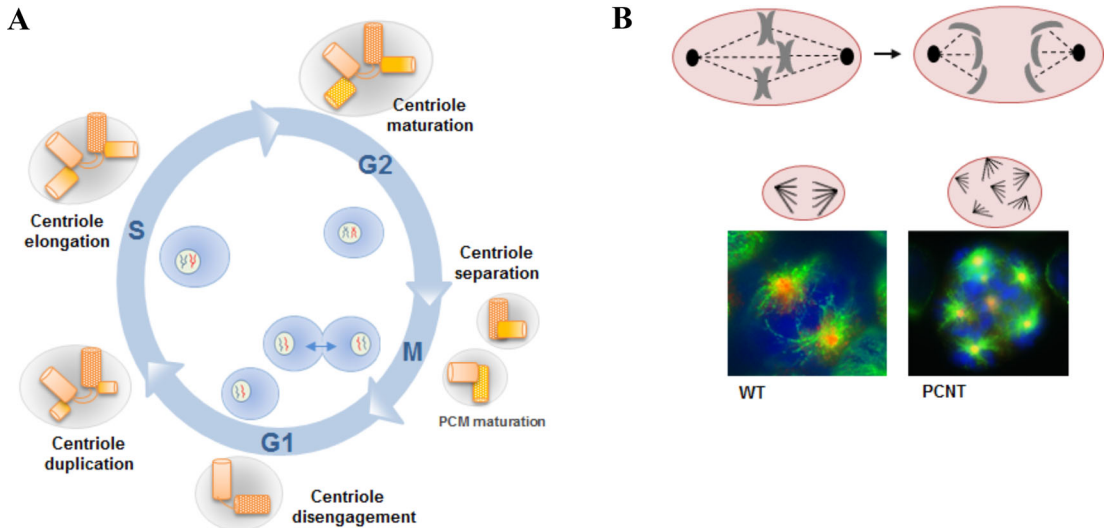

Figure 2. Centrioles, centrosome and mitotic microtubule organization. (**A**) The centriole cycle is intimately coordinated with the canonical cell cycle where centriolar division occurs during S-phase to ensure mature centrioles are available prior to the onset of mitosis. (**B**) A normal mitosis is depicted in the upper panel where the centrosomes are shown in black, the chromosomes in gray and the microtubule spindles as the dotted lines. Bi-polar spindle formation is an essential prerequisite to effective chromosome segregation during mitotic division. The lower panels show mitotic LCLs from a wild-type (WT) and *PCNT*-mutated individual. The spindles were detected using α-tubulin (green) whilst the centrosomes were stained using γ-tubulin (yellow-orange). The WT mitotic cell shows a bipolar spindle in contrast to the multipolar spindle from the PCNT-individual. (*Images courtesy of Dr. Iga Abramowicz*).

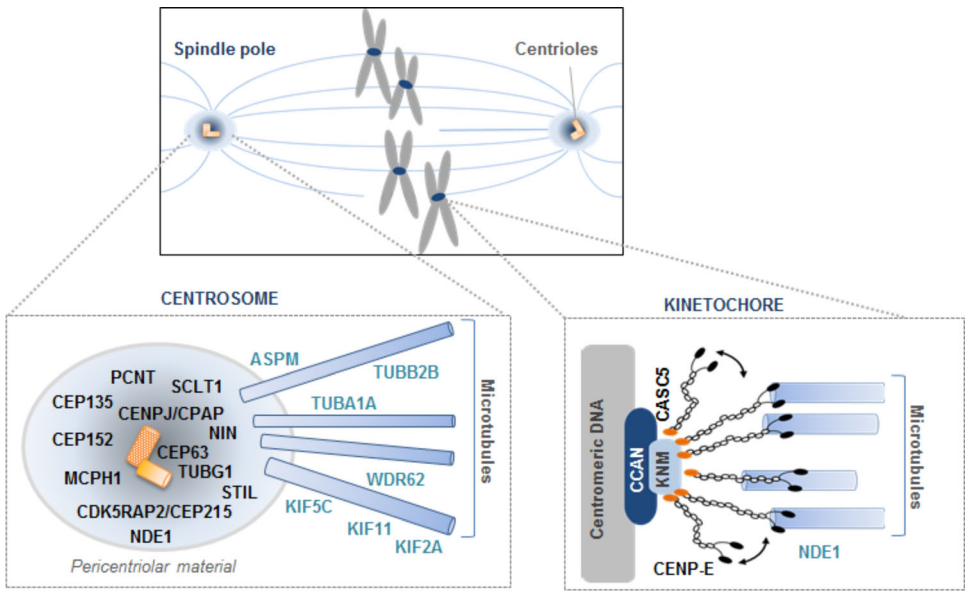

Figure 3. The distribution of genetic defects underlying congenital microcephaly attributable to the centrosome, spindle and kinetochore. The upper panel depicts a normal bipolar mitosis. For the centrosome panel, the proteins in black are centrosome-specific proteins whilst those in blue are spindle constituent and spindle-associated proteins associated with congenital microcephaly. For the kinetochore panel CENP-E is shown extending from the kinetochore via its large coiled-coiled region onto microtubules. The orange section of CENP-E denotes its kinesin motor domain. CCAN: constitutive centromere-associated network. KNM network: KNL1-NDC80 (CASC5)-MIND.

onset of the next mitosis [Hinchcliffe, 2001] (Fig. 2A). Currently, the working functional model to explain impaired neurogenesis in the context of a defect in a centrosomal protein is that these often result in supernumerary centrosomes, fragmented centrosomes and/or premature centriolar separation [Woods and Parker, 2013]. Consequently, these can result in deficits in mitotic spindle microtubule nucleation when establishing a bipolar spindle. Very often, multipolar spindles are observed in patient-derived cell lines representing a catastrophic consequence for mitosis, chromosome segregation and cell division [Rauch et al., 2008; Issa et al., 2013] (Fig. 2B). Such outcomes can result in permanent cell cycle arrest via activation of the spindle assembly checkpoint (SAC), as well as cytokinesis failure and subsequent apoptosis [Musacchio, 2011]. All of these impacts could limit neuroepithelial stem cell maintenance and expansion, as well as disrupt the important balance between cell division and differentiation (Fig. 1).

Defects in microtubule spindle components and spindle-associated proteins represent the most frequent underlying cause of congenital microcephaly described to date (Table I). One well known example is ASPM (*a*bnormal *s*pindle-like *m*icrocephaly-associated protein), a spindle binding protein that localizes to the pericentriolar matrix (PCM) of the centrosome at the onset of mitosis [Bond et al., 2002]. It has been shown that defects in ASPM function can result in altered spindle pole orientation in the developing neuroepithelium, thereby disrupting the balance between symmetric and asymmetric division of neuronal stem cells [Fish et al., 2006] (Fig. 1C). Indeed this has also recently been elegantly demonstrated in the developing brain of a mouse model of *Mcph1* (Microcephalin), a common cause of PM in humans [Gruber et al., 2011]. Defects in microtubule and cytoskeletal constituents (e.g., α- and β-tubulin) and even microtubule interacting proteins that regulate diverse processes such as microtubule formation, stabilization and depolymerization, can also result in congenital microcephaly [Morris-Rosendahl et al., 2008; Jaglin and Chelly, 2009; Romaniello et al., 2014] (Table I and Fig. 3). These defects are typically also associated with marked deficits in cortical development (e.g., lissencephaly, pachygyria, polymicrogyria) due to abnormalities in neuronal migration (e.g., KIF2A, KIF5C) [Poirier et al., 2013].

The Kinetochore and Spindles

Considering its role in microtubule capture and chromosome segregation, defects in kinetochore components as an underlying pathomechanism for congenital microcephaly are very much under-represented to date, compared to the spindle and centrosome (Table I and Fig. 3). Mutations in *BUB1B* cause mosaic variegated aneuploidy, an MPD associated with elevated cancer predisposition, particularly Wilms tumor [Matsuura et al., 2000; Hanks et al., 2004]. *BUB1B* encodes BUBR1, a SAC protein that localizes to the kinetochore of lagging chromosomes during mitosis [Bolanos-Garcia et al., 2009; Kiyomitsu et al., 2011]. SAC activation ensures all kinetochores have robust amphitelic microtubule attachments prior to segregation [Rudner and Murray, 1996; Musacchio, 2011].

A defect in *CASC5* was reported in three related families used to define the original MCPH4 locus [Jamieson et al., 1999; Genin et al., 2012]. *CASC5* encodes a component of the KMN Complex [*K*NL1-*M*is12 complex-*N*dc80], a kinetochore localizing multi-protein complex involved in microtubule stabilization and SAC silencing [Kiyomitsu et al., 2007]. Mirzaa et al. [2014] recently described the first example of a defect in a core kinetochore component as the underlying cause of MPD. They found defects in *CENPE*, the gene encoding the large kinetochore protein CENP-E which plays a vital role in microtubule capture during mitosis (Fig. 3). Cells from the affected individuals exhibited multiple interconnected mitotic abnormalities. It is likely that other kinetochore-associated defects await to be identified as novel causes of congenital microcephaly disorders, as PM and/or MPD.

DNA REPLICATION, CILIA FUNCTION AND MICROCEPHALY

Compared to other cell types, cell cycle length can be remarkably short in developing neuroprogenitors [Rakic, 1995]. Since these cells need to undergo rapid and temporally restricted expansion, efficient DNA replication is fundamental to ensure normal neuronal development. In fact, even within progenitor populations there appears to be significant variation in the duration of certain cell cycle phases; most notably G_1 and S-phase, depending upon the specific lineage commitment of the progenitors in question [Dehay et al., 2001; Dehay and Kennedy, 2007; Pilaz et al., 2009; Arai et al., 2011]. Therefore, genetic defects that can adversely impact upon the duration of these cell cycle phases could potentially have a dramatic effect upon the efficiency of cortical development.

Origin Licensing, G_1-S Transition, DNA Replication and S-Phase Progression

Recently, defects in multiple components of the origin recognition complex

Recently, defects in multiple components of the origin recognition complex [ORC], a multi-subunit complex that 'licenses' and thereby initiates DNA replication from mainly non-sequence specific discrete genomic regions, were identified in Meier-Gorlin syndrome (MGS).

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replication from mainly non-sequence specific discrete genomic regions, were identified in Meier-Gorlin syndrome (MGS) [Bicknell et al., 2011a,b; Guernsey et al., 2011] (Table II). MGS is an MPD associated with additional features including endochondral ossification abnormalities [Gorlin et al., 1975; Ahmad and Teebi, 1997; Bongers et al., 2005]. With respect to *ORC1*-mutated MGS, specific deficiencies in DNA origin licensing, DNA replication initiation, G_1-S transition and S-phase progression were catalogued in patient lymphoblastoid cell lines (LCLs), thus suggesting a pathomechanism based upon delayed DNA replication as underlying the clinical presentation [Bicknell et al., 2011b]. Furthermore, several *ORC1*-MGS mutations localized to the BAH (bromo adjacent homology) domain of ORC1 [Bicknell et al., 2011b]. This region was shown to bind histone H4-lysine-20-dimethylated (H4-K12-Me^2), and since H4-K12-Me^2 is enriched at two known human replication origins, it has been postulated that this domain is required for ORC-recruitment to origins [Noguchi et al., 2006; Kuo et al., 2012]. Collectively, these findings appear to support a model whereby a direct impact upon DNA replication kinetics, due to defects in components that initiate DNA replication, are associated with congenital microcephaly in the context of this MPD.

Recent findings suggest that there is more to this basic model. For example, pathogenic defects in MCM4, a core component of the replisome, have been identified in patients with adrenal insufficiency, growth restriction and a selective Natural Killer cell defect, although without any overt indication of congenital microcephaly [Casey et al., 2012; Gineau et al., 2012; Hughes et al., 2012]. Similarly, a polymerase-inactivating defect in the catalytic subunit (*POLD1*) of DNA polymerase δ, the lagging strand DNA polymerase, has been identified in an individual with a complex disorder involving lipodystrophy, deafness, hypogonadism and mandibular hypoplasia; again, without overt congenital microcephaly [Weedon et al., 2013].

Cilia-Function and S-Phase Entry

With respect to the impaired origin licensing and delayed S-phase kinetics observed in MGS, subsequent investigation provided evidence to suggest a more complex and multifaceted pathomechanism. Stiff et al. [2013] found that while MGS-causative defects were associated with reduced licensing of an ectopically supplied DNA replication origin, unexpectedly, some of these defects *did not* segregate with delayed S-phase progression in patient LCLs. Rather, all of these defects were associated with centriole-centrosome abnormalities, impaired cilia formation and consequently cilia-dependent signaling [Stiff et al., 2013]. Interestingly, ORC1 can localize to the centrosome in a Cyclin A-dependent manner and has previously been implicated in controlling centriole and centrosome copy number via interaction with Cyclin E [Hemerly et al., 2009]. Whether other ORC components have direct roles at the centrosome and/or cilia is not clear.

Cilia formation and function are vital for coordinating cell cycle entry from G_0 phase into G_1-S [Heldin and Westermark, 1999; Schneider et al., 2005]. Furthermore, cilia function is intimately associated with neuronal development [Spassky et al., 2008; Han and Alvarez-Buylla, 2010; Lee and Gleeson, 2011]. Delayed G_1-S transit appears to be a feature of MGS cells and this delay appears also to be dependent upon cilia-signaling [Stiff et al., 2013]. This is an intriguing result considering the importance of G_1 phase length in regulating the balance between neuroprogenitor stem cell regeneration and progenitor lineage commitment via differentiation [Dehay and Kennedy, 2007; Pilaz et al., 2009]. The cilia-dependent findings in MGS originating from defects in components with canonical roles in DNA replication adds an extra layer of complexity to our understanding of the molecular and cellular impacts that converge here to adversely affect normal neuronal development (and height attainment and endochondral ossification). Additionally, several ORC components have also been implicated in multiple post-mitotic neuronal functions, suggestive of additional roles outside of DNA replication origin licensing (reviewed in Kerzendorfer et al., 2013).

Is there precedence for centrosome-based cilia dysfunction in human congenital microcephaly disorders? Defects in *PCNT*, encoding the centrosomal protein Pericentrin, underlie the MPD microcephalic primordial osteodysplastic dwarfism type-II (MOPD-II) [Griffith et al., 2008; Rauch et al., 2008]. Impaired PCNT function is associated with cilia-dysfunction [Miyoshi et al., 2006, 2009; Mühlhans et al., 2011]. Multiple mutations in *POC1A* which encodes a centriole protein, have been identified in several MPD families [Shaheen et al., 2012]. Interestingly these defects were associated with centrosome fragmentation, microtubule spindle abnormalities (e.g., multipolar spindles) as well as cilia formation and signaling deficits [Shaheen et al., 2012]. In fact, ASPM has recently been associated with compromised WNT signaling which could also reflect an underlying problem in cilia function [Ponting, 2006; Buchman et al., 2011].

Therefore, it would appear that clinically relevant cilia abnormalities impacting on neurogenesis can originate from at least two routes; firstly, as a direct consequence of defects in proteins with known and/or probable roles in cilia formation which emanates from the centriolar-basal body, and secondly, from the likely secondary or indirect consequence of defects in proteins without prescribed roles in the cilium or in cilia formation, as in the case of the origin licensing components. The latter route may prove to be more widespread than currently appreciated.

THE DNA DAMAGE RESPONSE (DDR) AND MICROCEPHALY

The PI3-kinase-like family members ATM (*A*taxia *T*elangiectasia *M*utated) and ATR (*A*taxia *T*elangiectasia and *R*ad3-related) are the apical protein kinases of the DNA Damage Response (DDR) [Cimprich and Cortez, 2008;

Lavin, 2008; Maréchal and Zou, 2013; Shiloh and Ziv, 2013]. The DDR is a signal transduction cascade activated upon detection of DNA strand breaks that initiates and controls integrated responses to these damages [Sirbu and Cortez, 2013]. These responses include cell cycle checkpoint activation, DNA replication fork stabilization, engagement of DNA repair pathways and ultimately apoptosis, if the extent of damage precludes survival. ATM is activated by DNA double strand breakage (DSB) while ATR is activated by RPA-coated single stranded DNA (ssDNA) which can occur upon stalling of DNA replication forks or as an intermediate during certain DNA repair processes (e.g., DSB resection to facilitate homologous recombination repair). Both kinases largely function in a redundant manner in the DDR [O'Driscoll and Jeggo, 2008].

Congenital deficiency of ATM results in ataxia telangiectasia [A–T], a cerebellar neurodegenerative condition involving the progressive loss of Purkinje neurons specifically [Lavin, 2008]. Hypomorphic defects in *ATR* underlie Seckel syndrome; the archetypal MPD [O'Driscoll et al., 2003]. ATR has an obligate co-stabilizing binding partner called ATRIP (*ATR-i*nteracting *p*rotein), and defects herein have also been identified as a cause of Seckel syndrome [Ogi et al., 2012]. From an neuroanatomical perspective, impaired ATR-ATRIP function is most overtly associated with marked congenital microcephaly extending to −9 to −12 SD below the age related mean postnatally [Ogi et al., 2012]. Some problems in myelination and isolated structural abnormalities (e.g., absent pituitary fossa, agenesis of corpus callosum) have also been observed in these patients, although not consistently (Fig. 4A) [Ogi et al., 2012].

Hypomorphic defects in ATR underlie Seckel syndrome; the archetypal MPD. ATR has an obligate co-stabilising binding partner called ATRIP (ATR-Interacting Protein), and defects herein have also been identified as a cause of Seckel syndrome.

ATR plays a vital role in stabilising stalled DNA replication forks [Cimprich and Cortez, 2008]. Both ATM and ATR function in controlling dormant DNA replication origin firing and activation of G_1-S, intra-S and G_2-M cell cycle checkpoints. One of the first *bona fide* substrates identified and characterized for these kinases was p53 [Banin et al., 1998]. There are now many others [Matsuoka et al., 2007; Stokes et al., 2007]. Therefore, both kinases directly signal to the apoptotic machinery via this and other routes [Roos and Kaina, 2006]. Loss of ATR function is associated with elevated replication fork collapse and consequently DSB formation, therefore triggering an ATM-dependent DDR cascade. This was elegantly demonstrated as a physiological consequence of hypomorphic ATR-ablation in a mouse model of *ATR*-mutated Seckel syndrome ($Atr^{S/S}$) [Murga et al., 2009]. Embryos of the $Atr^{S/S}$ animal exhibited massively elevated spontaneous levels of replicative stress-induced DNA damage and apoptosis, even in the developing neuroepithelium [Murga et al., 2009]. The surviving animals exhibited craniofacial abnormalities (receding forehead, micrognathia), growth restriction and congenital microcephaly reminiscent of the *ATR*-mutated Seckel syndrome individuals, consistent with the concept of intrauterine programming [Murga et al., 2009; O'Driscoll, 2009].

Defective DDR, Apoptosis and Microcephaly

Conditional *Cre*-restricted mouse models have shown that the functional interrelationships between in the apical DDR kinases are more complex when considering brain development. For example, conditional *Atr* ablation was unexpectedly found to impact relatively late upon neurogenesis, and then only in certain progenitor populations [Lee et al., 2012b]. Rapid proliferation of granule neurons within the embryonic cerebellar external germ layer (EGL) occurs in response to sonic hedgehog (Shh), whose mitogenic potential is realized via cilia [Spassky et al., 2008]. *Atr*-deficient EGL cells underwent p53-independent proliferation arrest, while other areas underwent p53-dependent apoptosis [Lee et al., 2012b]. Furthermore, co-incident inactivation of *Atm* was unexpectedly found not to exacerbate *Atr* loss in the brain, suggesting a non-overlapping role for each kinase in this developmental context [Lee et al., 2012b].

Topoisomerase II binding protein I (TopBP1) is essential for DNA replication and cell cycle checkpoint activation [Sokka et al., 2010]. It also plays a key role in activating ATR kinase [Kumagai et al., 2006]. Interestingly, conditional progenitor-restricted deletion of *Topbp1* was found to be essential for early progenitor genome stability and survival, but not, unexpectedly, for replication *per se* [Lee et al., 2012a]. Furthermore, this impact was found to be p53-dependent whilst Atm-independent [Lee et al., 2012a]. Atm has previously been shown to be an important mediator of p53-dependent DSB-induced apoptosis in the nervous system [Herzog et al., 1998]. Collectively, these findings highlight the importance of the cell-specific context within the brain, along with the nature of the genomic instability (e.g., replication fork-dependent), as being fundamental to the precise impacts of defects in DDR-signaling controllers such as Atr, Atm and Topbp1, in the developing mouse brain at least.

DEFECTIVE DNA DOUBLE STRAND BREAK [DSB] REPAIR AND MICROCEPHALY

Non-homologous DNA End-Joining (NHEJ) and Homologous Recombination Repair (HRR) are the core DSB repair pathways [O'Driscoll and Jeggo, 2006; Moynahan and Jasin, 2010; Deriano and Roth, 2013]. Interestingly,

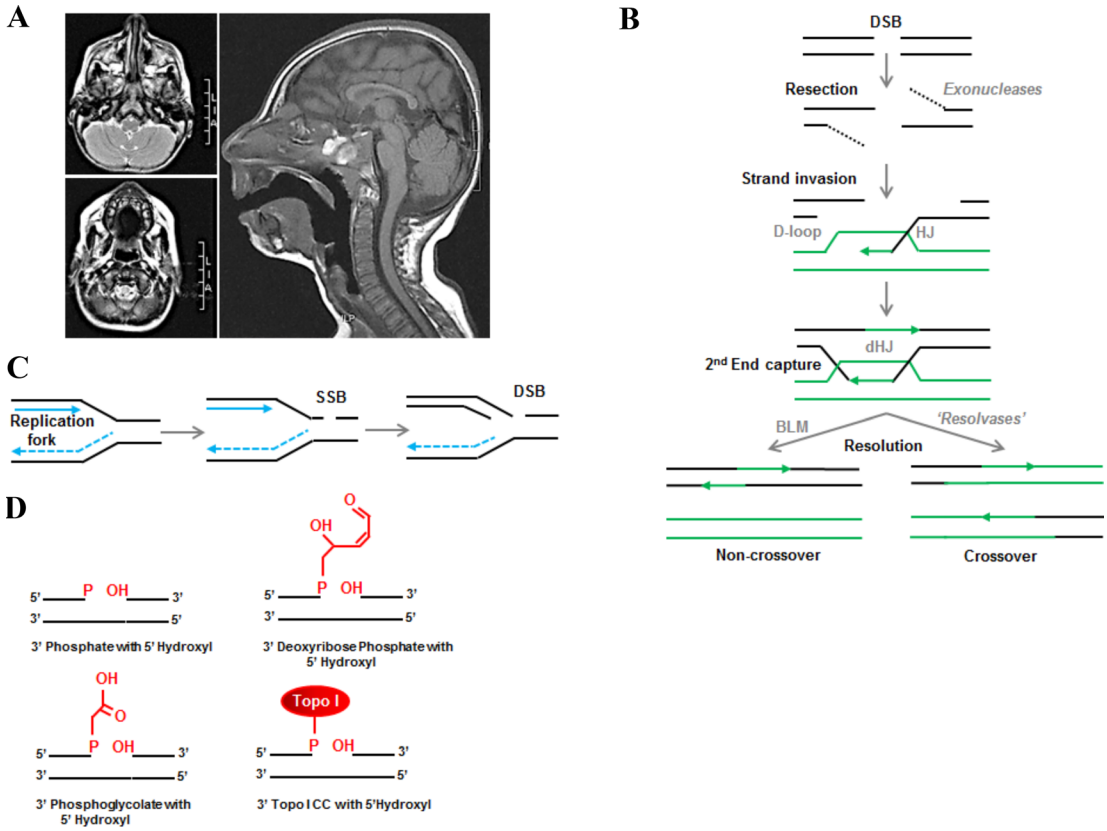

Figure 4. Defective DDR and DNA repair as a cause of congenital microcephaly. (**A**) Neuroanatomical images of the *ATRIP*-mutated Seckel syndrome individual described by [Ogi et al., 2012]. The patient is severely microcephalic [−10 SD] with evidence of an abnormally shaped pituitary (without an obvious fossa). (*Images courtesy of Dr. Margaret Barrow*). (**B**) Homologous recombination repair (HRR) from a DSB is initiated by exonuclease-mediated resection of the DSB ends. These RPA-coated single stranded overhangs are then bound by RAD51 generating a filament structure that can invade the sister chromatid to form a D-loop. The crossover point is referred to as a Holliday Junction (HJ). Template driven replication occurs and is followed by second end capture to generate a crossover containing a double HJ (dHJ). This intertwined molecule requires resolution which can occur via BLM helicase-dependent route, which generates a non-crossover product, or, a nuclease-dependent route to generate a cross-over. The nucleases that can act on this structure are termed "resolvases." (**C**) An on-going DNA replication fork is shown with the parental DNA in black and the newly replicated DNA in blue. If this structure collides with a DNA single strand break (SSB) the resultant product can contain a DNA double strand break (DSB). This situation requires the coordinate action of DDR mechanisms and distinct DNA repair pathways. The same situation can also occur if an active or moving transcription fork was to collide with a SSB. (**D**) Some of the damaged or modified DNA strand ends that can occur following oxidative damage to DNA. The normal end polarity of a 5′ phosphate and 3′ hydroxyl group must be restored in order to allow enzymatic ligation of a break. Topo I denotes a stabilized Topoisomerase I cleavable complex (CC) which is a normal intermediate in Topo I's action on DNA. This enzyme normally introduces a SSB to release torsional tension ahead of on-going replication and transcription forks. These breaks are normally dealt with by the SSBR machinery.

congenital defects in DSB repair mechanisms are frequently associated with congenital microcephaly [O'Driscoll and Jeggo, 2008]. Mouse studies suggest that both pathways are essential for developmental viability in mammals; an indication that substantial levels of spontaneous DSBs can be generated during development [Symington and Gautier, 2011]. NHEJ involves direct religation of DSBs, sometimes necessitating processing of damaged base and/or sugar moieties at DSB termini prior to ligation [Deriano and Roth, 2013]. This processing can potentially result in the loss of genetic material. HRR on the other-hand requires the presence of a sister chromatid to act as a template for repair via strand invasion from one end of the DSB during Holliday Junction (HJ) formation [Heyer et al., 2010] (Fig. 4B). Therefore, this pathway has been assumed to only be operational during cell cycle phases where a sister chromatid is present (i.e., late S-G_2-M) [Symington and Gautier, 2011].

Non-Homologous DNA End-Joining [NHEJ]

NHEJ is required for immunoglobin and T cell receptor generation via the V(D)J and Class Switch Recombination mechanisms [Gellert, 2002]. Therefore, congenital defects in NHEJ pathway components are also frequently associated with variable immunodeficiency, ranging from variable/combined immunodeficiency to severe combined immunodeficiency [O'Driscoll and Jeggo,

2006]. To date, human defects have been identified in *PRKCD* encoding DNA-PKcs, *DCLRE1C* encoding ARTEMIS, *LIG4* encoding DNA ligase IV and the genes encoding XRCC4 and XLF/Cernunnos [Moshous et al., 2001; O'Driscoll et al., 2001; Buck et al., 2006; van der Burg et al., 2009; Woodbine et al., 2013; Murray et al., 2014; Shaheen et al., 2014]. Interestingly, ARTEMIS-defective patients, uniquely in this context, do not appear to exhibit microcephaly, in contrast to the other genes [Moshous et al., 2001]. This may be a consequence of the magnitude and nature of their DSB repair; ARTEMIS-defective cells exhibit a specific DSB repair in heterochromatin; an ATM-dependent process [Riballo et al., 2004].

Homologous Recombination Repair

By contrast to NHEJ, congenital defects in core components of the HRR machinery are still relatively rare in humans, although some isolated germ-line defects have been described [reviewed in O'Driscoll, 2012]. Recent examples include *XRCC2* in a patient exhibiting fanconi anemia (FA), *BRCA1* in a woman with early onset ovarian cancer and microcephaly, and *BRCA2* in the context of MPD [Shamseldin et al., 2012; Domchek et al., 2013; Shaheen et al., 2014]. FA patients frequently exhibit congenital microcephaly although bone marrow failure and acute myeloid leukaemia development are the typically invariant features of this condition [Kee and Andrea, 2012]. The FA pathway is functionally integrated with HRR and defects in other core HRR components have emerged clinically as FA; the classical example being *BRCA2* mutations in FA individuals of the FANC-D1 complementation group [Moldovan and D'Andrea, 2009; Kim and D'Andrea, 2012]. Other examples include *RAD51C* (FANC-O), *PALB2*, encoding the BRCA2 interacting protein (FANC-N) and *SLX4* (FANC-P), encoding a component of the SLX4-SLK1 HJ resolving endonuclease (Fig. 4B). Interestingly, germ-line mutations (heterozygous) in the HRR genes *RAD51C* and *RAD51D* have been identified in breast and ovarian cancer cohorts [Meindl et al., 2010; Loveday et al., 2011, 2012; Shaheen et al., 2014]. This suggesting that HRR defects can have a highly variable clinical presentation.

Helicases and Nucleases

The Bloom syndrome helicase BLM [*RECQL3*] plays an important role in HJ resolution, and congenital microcephaly can be marked in this condition [Hickson, 2003; Croteau et al., 2014] (Fig. 4B). Warsaw breakage syndrome, a disorder of severe congenital microcephaly, growth retardation and chromosomal instability collectively reminiscent of FA and the cohensinopathies, was found to result from a mutation in another ATP-dependent DNA helicase, CHLR1 (*DDX11*) [van der Lelij et al., 2010; Capo-Chichi et al., 2013]. This helicase has been implicated in chromosome cohesion and in the recovery of replicating cells from DNA damage [Shah et al., 2013]. DNA2 is a helicase-nuclease implicated in Okazaki fragment processing and the processing of abnormally structured replication forks (e.g., aberrant recombination intermediates) [Mimitou and Symington, 2009; Kang et al., 2010]. A truncating *DNA2* mutation has recently been identified in a consanguineous MPD family [Shaheen et al., 2014]. This category of defect reinforces the concept that an impaired ability to process DNA breaks during DNA replication may represent a contributing pathomechanism to congenital microcephaly.

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Prior to RAD51-mediated strand invasion to establish HJs during HRR, DSBs in S-G_2 undergo resection (Fig. 4B). Various exonucleases have been implicated in this process, one of best known being the MRE11-RAD50-NBN (M-R-N) complex [Symington and Gautier, 2011]. ATM directly phosphorylates each component of this complex following DSB formation. Mutations in all M-R-N components have now been identified in clinically overlapping human disorders exhibiting congenital microcephaly. Defects in *NBN* encoding Nibrin/NBS1 cause Nijmegen breakage syndrome (NBS), a disorder of congenital microcephaly, growth restriction and haematological malignancy [Carney et al., 1998; Varon et al., 1998]. Defects in *RAD50* have been shown to cause an NBS-like disorder, whilst mutations in *MRE11* are associated with a clinical spectrum ranging from A-T-Like Disorder (A-T-LD) without overt microcephaly to an NBS-like condition, probably reflective of different genotype-phenotype impacts [Stewart et al., 1999; Waltes et al., 2009; Matsumoto et al., 2011]. CtIP (CTBP-interacting protein) is another of these exonucleases implicated in DSB resection [Mimitou and Symington, 2009]. CtIP is encoded by *RBBP8* (*r*etino*b*lastoma *b*inding *p*rotein *8*), wherein a hypomorphic mutation has been described in a Seckel syndrome kindred [Qvist et al., 2011]. Therefore, hypomorphic defects in various exonucleases that function during specific stages of DSB repair are emerging as an underlying cause of congenital microcephaly.

DSB Repair Pathways in the Developing Brain: Spatial Regulation and Checkpoint Activation

As mentioned above, HRR is by its nature of requiring a homologous DNA strand as a template for repair, restricted to specific phases of the cell cycle where sister chromatids are available (i.e., S-G_2-M) (Fig. 4B). Therefore, tissues with a very high replicative index would be expected to rely heavily on this pathway to repair DSBs, compared to non-replicative post-mitotic tissues. Studies using knockout embryonic mouse models of NHEJ and HRR have suggested that both these DSB repair pathways may have distinct spatiotemporal functionality during neuronal development [Orii et al., 2006]. Defective HRR (*Xrcc2*)-induced apoptosis appeared to occur predominantly within the proliferating neuronal precursors of the ventricular zone (VZ) [Orii et al., 2006] (Fig. 1A). In contrast, defective NHEJ (*Lig4*)-induced apoptosis appeared restricted to the postmitotic differentiating neurons of the subventricular zone (SVZ) [Orii et al., 2006]. Work using ionising radiation (IR)-induced DSBs in a hypomorphic *Lig4* embryonic mouse model ($Lig4^{Y288C}$) has further developed this concept by providing evidence for a functional, but relatively insensitive, G2-M cell cycle checkpoint in the VZ-SVZ [Gatz et al., 2011]. This insensitive checkpoint allows cells with low numbers of DSBs to transit from the VZ-SVZ to the intermediate zone (IZ)-cortical plate (CP) region where they then die by apoptosis [Gatz et al., 2011]. Interestingly, work in somatic cells has shown that the DSB-induced G2-M cell cycle checkpoint can be quite an ineffective block to preventing cells with modest levels of DSBs from entering mitosis [Lobrich and Jeggo, 2007].

Origins of Endogenous DNA Breaks in the Developing Brain

A growing body of evidence now indicates that the inability to repair DSBs because of defects in NHEJ and/or HRR clearly adversely impacts upon normal neurogenesis often resulting in congenital microcephaly [O'Driscoll and Jeggo, 2008; O'Driscoll, 2012]. There has been much speculation as to what precisely could be the origin of endogenous DSBs during neurogenesis. One suspect, because of the high oxygen consumption and metabolic rate of neurons and their supportive cells, is endogenously generated reactive oxygen species (ROS)-induced oxidative DNA damage [Caldecott, 2008]. This can result in base and ribose sugar backbone damage/modifications, abasic apurinic/apyrimidinic (AP) site formation (i.e., base loss) and even overt single strand break (SSB) formation. These lesions are rapidly dealt with by the coordinated action of the base excision repair (BER) and single strand break repair (SSBR) pathways [Caldecott, 2008]. If an active DNA replication or transcription fork encounters an SSB there is an elevated risk of consequent DSB formation (Fig. 4C).

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Congenital defects in certain SSBR components have been identified in humans, but these are usually associated with slowly progressive cerebellar degeneration, ultimately presenting with ataxia and peripheral neuropathy-disorders, rather than congenital microcephaly [Caldecott, 2008; O'Driscoll, 2012]. It is likely that active and complementary DNA-repair pathways co-ordinately play a protective role during neurogenesis in this context. One example of a defect in a core SSBR component where this complementarity may be compromised is polynucleotide kinase-phosphatase (PNKP). Multiple novel mutations in *PNKP* were recently described in several individuals exhibiting severe primary microcephaly, developmental delay, hyperactivity and seizures (MCSZ) [Shen et al., 2010]. Interestingly, PNKP's dual kinase and phosphatase activity, which is essential for repair of the aberrantly modified termini at strand breaks, is also implicated in NHEJ (Fig. 4D) [Caldecott, 2002; Koch et al., 2004; Weinfeld et al., 2011]. Therefore, the marked congenital microcephaly in MCSZ may be more reflective of combined attenuation of both SSBR and DSB repair pathways.

CONCLUDING REMARKS

Congenital microcephaly has a complex underlying genetic basis, as demonstrated by the selection of defects discussed here. An inability to divide and differentiate effectively resides at the center of this and there are multiple routes through which these processes can be disrupted. The growing number of causative genetic defects described for congenital microcephaly consolidates the importance of mitotic spindle organization and centrosome stability in enabling the execution of precise chromosome segregation during mitosis. Novel causative genetic defects also provide evidence that perturbations of other cell cycle phases are also relevant to normal neurogenesis, for example, DNA replication origin licensing, G1-S entry, DNA synthesis and efficient S-phase progression. The importance and integration of functional cilia-signaling in the context of regulated cell cycle entry and progression cannot be overstated. Finally, defects in multiple components of the complex pathways that control genome stability and integrity via signal transduction and repair processes are additional important contributors to congenital microcephaly.

Without doubt, the advent of exome sequencing has greatly facilitated the identification of novel microcephaly-causing defects, further developing our understanding of the molecular basis of this abnormality. This trend is likely to continue. The consequent challenge in

future will be functionally validating and determining the pathogenicity of the multitude of candidate variants derived from such approaches. This issue is compounded by the multigenic occurrence of variants, which is still likely an under-appreciated and under-reported situation at present [Agha et al., 2014]. Functional cellular biology using patient-derived cell lines, model cell systems (siRNA, shRNA, cDNA complementation strategies) and even iPS from patients' cells, will likely continue to remain the basic cornerstone approaches in helping to meet this challenge. The growing use of more cost-effective and rapidly growing model organisms in this area, such as zebrafish, is also likely to continue [Novorol et al., 2013]. For now, mouse models remain the effective 'gold standard' for neurogenic-microcephaly research, but the potential offered by the fascinating cerebral organoid system represents a very exciting future prospect [Lancaster et al., 2013].

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