



Dissecting the Genetic and Etiological Causes of Primary Microcephaly

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Autosomal recessive primary microcephaly (MCPH; “small head syndrome”) is a rare, heterogeneous disease arising from the decreased production of neurons during brain development. As of August 2020, the Online Mendelian Inheritance in Man (OMIM) database lists 25 genes (involved in molecular processes such as centriole biogenesis, microtubule dynamics, spindle positioning, DNA repair, transcriptional regulation, Wnt signaling, and cell cycle checkpoints) that are implicated in causing MCPH. Many of these 25 genes were only discovered in the last 10 years following advances in exome and genome sequencing that have improved our ability to identify disease-causing variants. Despite these advances, many patients still lack a genetic diagnosis. This demonstrates a need to understand in greater detail the molecular mechanisms and genetics underlying MCPH. Here, we briefly review the molecular functions of each MCPH gene and how their loss disrupts the neurogenesis program, ultimately demonstrating that microcephaly arises from cell cycle dysregulation. We also explore the current issues in the genetic basis and clinical presentation of MCPH as additional avenues of improving gene/variant prioritization. Ultimately, we illustrate that the detailed exploration of the etiology and inheritance of MCPH improves the predictive power in identifying previously unknown MCPH candidates and diagnosing microcephalic patients.

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INTRODUCTION

Autosomal recessive primary microcephaly (MCPH) is a rare, heterogeneous disorder characterized by an occipitofrontal diameter >2 or 3 standard deviations below the mean at birth, after accounting for sex and ethnicity. MCPH patients typically have a simplified cerebral cortical gyral pattern (lissencephaly) although overall brain architecture is generally normal. MCPH is also frequently associated with other clinical features such as intellectual impairment, short stature, and mild seizures—since these features frequently overlap with other syndromes, it is likely that MCPH is part of a disease spectrum. The rate of incidence varies between 1 in 10,000, in populations where consanguineous marriages are common, and 1 in 250,000 in non-consanguineous populations (1, 2). Although there are some examples of dominantly inherited primary microcephaly, it is typically inherited in an autosomal recessive manner.

There are currently 25 MCPH-associated genes listed in the Online Mendelian Inheritance in Man (OMIM) database (accessed as of April 2020): *MCPH1*, *WDR62*, *CDK5RAP2*, *KNL1*, *ASPM*, *CENPJ*, *STIL*, *CEP135*, *CEP152*, *ZNF335*, *PHC1*, *CDK6*, *CENPE*, *SASS6*, *MFSD2A*, *ANKLE2*,

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CIT, *WDFY3*, *COPB2*, *KIF14*, *NCAPD2*, *NCAPD3*, *NCAPH*, *NUP37*, and *MAP11* (MCPH1–25, respectively) (**Table 1**). Some of these genes cause microcephaly in combination with other disease features (syndromic microcephaly), however, we will focus on their involvement in primary microcephaly in this review. Because of its heterogeneous nature, diagnosing patients can be a challenging endeavor. Gene panel testing is effective at identifying the more common genetic variants that cause MCPH but becomes impractical with the continual updates required as new genes and variants are discovered. For instance, a recent case report describes a patient with primary microcephaly in which several gene panels were unable to find the disease-causing variant, including the autosomal recessive primary microcephaly Tier 2 sequencing and deletion/duplication panel that screens for several MCPH genes (67). Once whole exome sequencing (WES) was performed on this individual, a homozygous variant in the DNA damage response gene, *TTI2*, was found, representing a novel MCPH locus. Accordingly, it then makes sense to turn to exome or whole genome sequencing (WGS) in order to diagnose patients; however, although these techniques give us a more complete picture of the genome, they do not always immediately provide answers. Exome sequencing of MCPH patients has been shown to have a diagnostic yield of only 29%, with a particular bias for identifying *ASPM* mutations (68). Although that number does increase to about 50% with WGS, this still leaves half of MCPH patients and their families without answers (69). One issue is that WES or WGS typically results in thousands of variants, making the variant prioritization process a daunting endeavor. Therefore, while there is an obvious need to improve our analysis of WES and WGS in order to identify disease-causing variants, it is also necessary to improve our understanding of the causes of MCPH to be able to make new gene–disease associations.

The overall goal of this review is to describe different aspects of MCPH that impact our ability to discover new genes associated with MCPH and, consequently, to diagnose patients. To achieve this goal, we first demonstrate that MCPH arises from perturbations in cell cycle regulation by briefly highlighting the cellular role of microcephaly-associated proteins. Then, we discuss the genetics and evolution of these genes as further considerations in variant prioritization in patients. Throughout this, we provide examples that exemplify how a thorough understanding of the etiology and genetics of disease allows us to identify new disease-causing candidates. Finally, we will comment on a major question in the field—based on what we know about the etiology and genetics of MCPH, why do mutations result in a brain-specific phenotype? Altogether, we illustrate that multidisciplinary approaches facilitate the prioritization of MCPH variants in patients with unknown genetic causes.

DISSECTING THE ETIOLOGY OF PRIMARY MICROCEPHALY

MCPH is typically caused by a reduction in the number of neurons in the developing neocortex. Neurons are derived from

apical progenitor cells (APs) in the ventricular zone (VZ) of the neocortex (70). In a brief overview, APs divide symmetrically to produce two progenitor cells in the proliferative phase. At the onset of neurogenesis, symmetric cell divisions are favored in order to generate a large pool of progenitor cells; the size of this pool is a good indicator of eventual brain size (**Figure 1A**) (71). In early neurogenesis phases, APs begin to express glial markers and adopt a radial glial cell (RG) fate; these cells have a highly polarized architecture and are able to divide symmetrically to generate more RGs or young neurons (**Figure 1B**) (70, 72). As neurogenesis continues, RGs begin to favor asymmetric cell divisions in order to generate more neurons and a secondary progenitor cell, termed basal progenitors (BPs) that localize to the subventricular zone (**Figure 1C**) (70). BPs serve to amplify the number of neurons that are formed per AP division.

Based on this developmental model, there are a number of reasons why fewer neurons are produced in MCPH patients. There can be increased cell death of these neurons, an imbalance in the ratio of progenitor to differentiating cells (i.e., changes in asymmetric vs. symmetric cell divisions), changes in the timing of the cell divisions, or abnormal differentiation. However, although each of the MCPH-associated genes affects neuronal population size given their involvement in microcephaly, these genes are involved in a number of different cellular processes that seem rather unconnected. For instance, MCPH genes are involved in centriole biogenesis and regulation, DNA replication and division, cell division, signaling, mitotic spindle orientation, chromosomal condensation, DNA damage responses, microtubule dynamics, and transcriptional control (**Figure 2**). Despite these seemingly diverse cellular functions, there appears to be a common disease mechanism linking each of these processes—mutations in MCPH genes disrupt the timing of the neurogenic program. This can be modeled using the cell cycle exit fraction, which is the ratio of cells that take on a differentiated fate (i.e., become neurons) vs. those that remain in a proliferative state (73).

To give context to readers that may not be familiar with the cellular function of MCPH genes, this review will begin by demonstrating that mutations in the highlighted genes affect cell cycle timing in some way, effectively increasing the cell cycle exit fraction, indicating that MCPH is a disease arising from cell cycle dysregulation (**Figure 2**). The cell cycle checkpoints ensure that the cell is appropriately prepared for cell division; they are activated by events such as DNA damage (G2 checkpoint), unattached kinetochores (M checkpoint), limited resources (G1 checkpoint), and/or signaling cues (G1 checkpoint) [reviewed in Pucci et al. (74)]. These checkpoints delay the onset of the next phase to permit the cell to correct any errors that arise. If errors can be repaired, the cell continues to progress through the cell cycle, but if errors are incapable of being fixed, often due to mutations, the cell will often undergo apoptosis to prevent errors from being transmitted (74). Accordingly, not only do ongoing cell cycle delays produce fewer neurons, it is compounded by apoptosis, which further reduces the number of progenitors and neurons within the neocortex. Therefore, a disruption in cell cycle timing appears to be the common mode of pathogenesis underlying MCPH. Although we primarily focus on the described

TABLE 1 | Overview of OMIM-listed MCPH genes (as of April 2020).

Gene name	Description	Cell process	Disease ID	Inheritance	Mode of inactivation	Other clinical features	Other associated conditions	Orthologs
CENTRIOLE BIOGENESIS								
<i>CENPJ</i>	Centromere protein J	Centriole biogenesis PCM tethering	MCPH6	AR	Non-sense, frameshift, missense (non-conservative), splicing (3–7)	Facial dysmorphism, developmental delay, joint stiffness, seizures, intellectual disability, cortical malformations, motor problems (3–5)	Seckel syndrome (8)	<i>sas-4</i> (<i>C. elegans</i>), <i>Dsas-4</i> (<i>Drosophila</i>), <i>cenpj</i> (mouse, zebrafish)
<i>STIL</i>	SCL/TAL-interrupting locus	Centriole biogenesis	MCPH7	AR	Non-sense, frameshift, missense (non-conservative), splicing (4, 9–11)	Short stature, seizures, intellectual disability, cortical malformations, visual impairment, motor problems, pre-mature death (3, 4, 11, 12)		<i>sas-5</i> (<i>C. elegans</i>), <i>ana2</i> (<i>Drosophila</i>), <i>stil</i> (mouse, zebrafish)
<i>CEP135</i>	Centrosomal protein 135	Centriole biogenesis	MCPH8	AR	Non-sense, frameshift, splicing (4, 13, 14)	Facial dysmorphism, intellectual disability, cortical malformations, short stature, motor problems, hearing loss (4, 13, 14)		<i>cep135</i> (<i>Drosophila</i> , mouse, zebrafish), <i>bld10</i> (<i>Chlamydomonas</i>)
<i>CEP152</i>	Centrosomal protein 152	Centriole biogenesis	MCPH9	AR	Missense (non-conservative), non-sense, frameshift, splicing (4, 6, 15, 16)	Cortical malformations, facial dysmorphism, intellectual disability, motor problems (4, 11, 16)	Seckel syndrome (15)	<i>asterless</i> (<i>Drosophila</i>), <i>cep152</i> (mouse, zebrafish)
<i>SASS6</i>	Spindle assembly abnormal 6	Centriole biogenesis	MCPH14	AR	Missense (non-conservative) (17)	Intellectual disability, cortical malformations (17)		<i>sas-6</i> (<i>C. elegans</i> , <i>Drosophila</i>), <i>bld12p</i> (<i>Chlamydomonas</i>), <i>sass6</i> (mouse, zebrafish)
MICROTUBULE DYNAMICS								
<i>WDR62</i>	WD repeat domain 62	Centriole biogenesis PCM scaffold Microtubule nucleation Spindle orientation	MCPH2	AR	Non-sense, frameshift, missense (non-conservative), splicing (4, 6, 18–23)	Intellectual disability, seizures, motor problems, facial dysmorphism, cortical malformations, developmental delay (3, 18, 19, 22, 23)	Polymicrogyria (18, 22)	<i>H24G06.1</i> (<i>C. elegans</i>), <i>wdr62</i> (<i>Drosophila</i> , mouse, zebrafish)
<i>CDK5RAP2</i>	CDK5 regulatory subunit-associated protein 2	PCM scaffold Microtubule nucleation Centriolar engagement Cytokinesis Spindle orientation	MCPH3	AR	Non-sense, frameshift, splicing, missense (non-conservative) (7, 24–29)	Hearing loss, leukemia, intellectual disability, short stature, pigmentation abnormalities, facial dysmorphism, cortical malformations (4, 24–31)	Seckel syndrome (24)	<i>spd-5</i> (<i>C. elegans</i>), <i>cnn</i> (<i>Drosophila</i>), <i>cdk5rap2</i> (mouse, zebrafish)
<i>KNL1</i>	Kinetochore scaffold 1	Kinetochore attachment Mitotic checkpoint complex regulator	MCPH4	AR	Splicing, frameshift, missense (non-conservative) (32, 33)	Intellectual disability, cortical malformations, facial dysmorphism, short stature (32, 34, 35)		<i>knl-1</i> (<i>C. elegans</i>), <i>knl1</i> (mouse, zebrafish)

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TABLE 1 | Continued

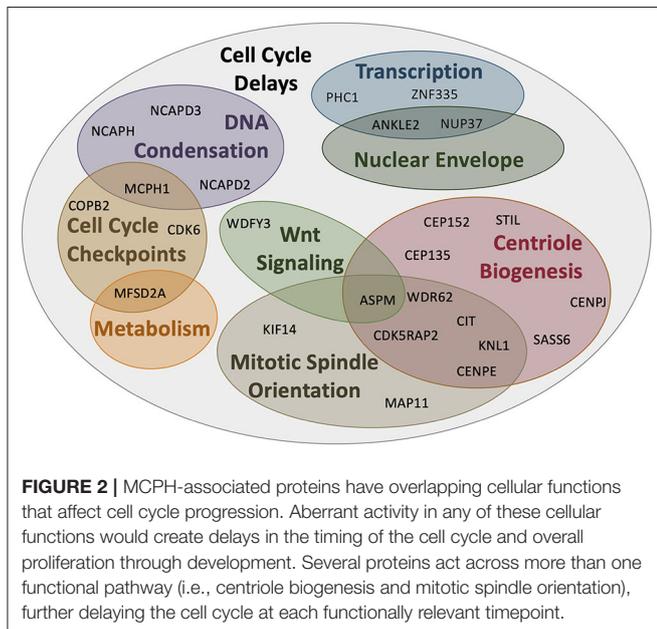
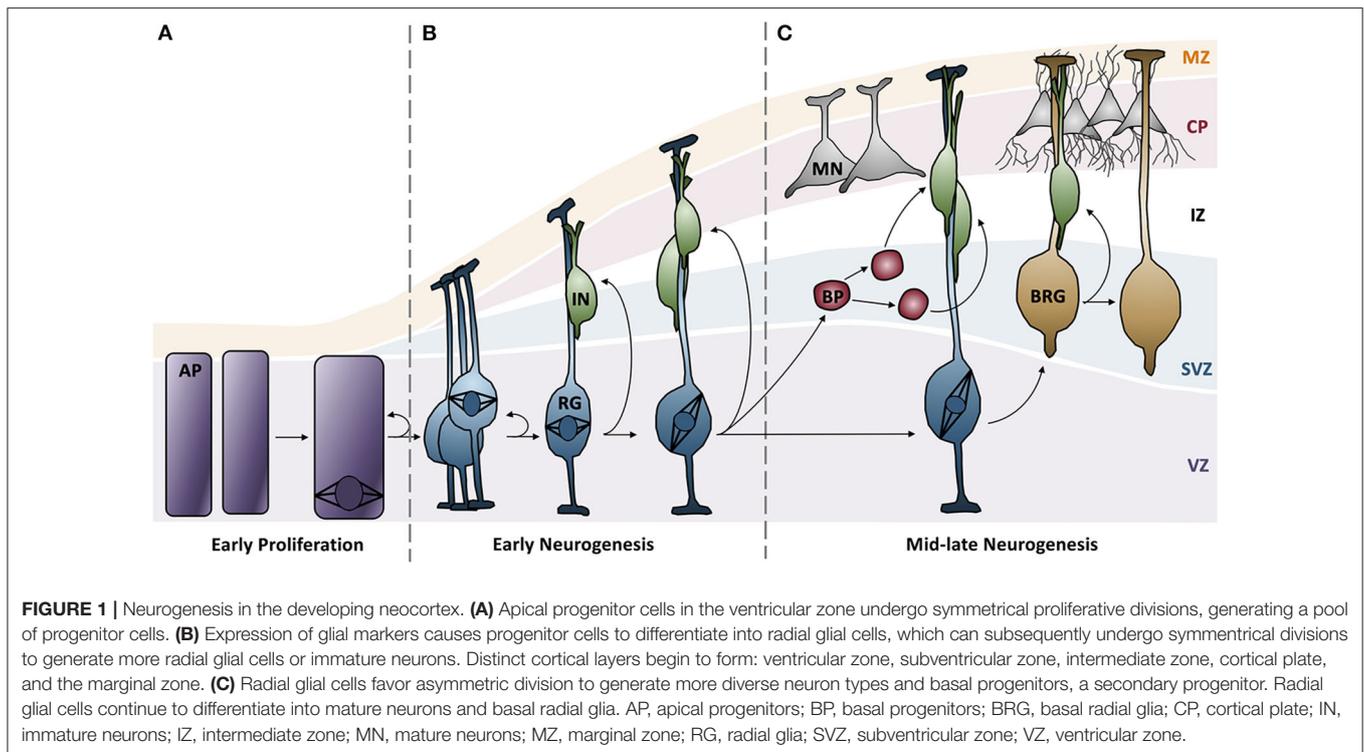
Gene name	Description	Cell process	Disease ID	Inheritance	Mode of inactivation	Other clinical features	Other associated conditions	Orthologs
<i>ASPM</i>	Abnormal spindle microtubule assembly	Wnt signaling Centriole biogenesis Spindle orientation Cytokinesis	MCPH5	AR	Non-sense, deletion, frameshift, missense (non-conservative), splicing, structural variant (3, 4, 6, 10, 36–40)	Short stature, cortical malformations, heart defects, facial dysmorphism, intellectual disability, pigmentation abnormalities, motor problems, seizures (3, 4, 38–40)		<i>aspm-1</i> (<i>C. elegans</i>), <i>asp</i> (<i>Drosophila</i>), <i>aspm</i> (mouse, zebrafish)
<i>CENPE</i>	Centromere protein E	Kinetochore attachment Mitotic checkpoint complex regulator	MCPH13	AR	Missense (non-conservative) (41)	Facial dysmorphism, seizures, heart defects, intellectual disability, pre-mature death, cortical malformations, motor problems (41)	Microcephalic primordial dwarfism (41)	<i>cana/cmet</i> (<i>Drosophila</i>), <i>cenpe</i> (mouse, zebrafish)
<i>CIT</i>	Citron rho-interacting serine/threonine kinase	Microtubule nucleation Cytokinesis Spindle orientation	MCPH17	AR	Missense (non-conservative), splicing, frameshift, non-sense (4, 42–44)	Short stature, intellectual disability, cortical malformations, pre-mature death (42–45)		<i>W02B8.2</i> (<i>C. elegans</i>), <i>sticky</i> (<i>Drosophila</i>), <i>cit</i> (mouse), <i>cita/citb</i> (zebrafish)
<i>KIF14</i>	Kinesin 14	Cytokinesis Microtubule network stabilizer	MCPH20	AR	Non-sense, splicing, missense (non-conservative), frameshift (46, 47)	Intellectual disability, speech impairment, developmental delay, motor problems, spasticity, facial dysmorphism, blindness, ADHD, hypotonia (46, 47)	Meckel syndrome (48)	<i>kfp-6</i> (<i>C. elegans</i>), <i>nebbish</i> (<i>Drosophila</i>), <i>kif14</i> (mouse, zebrafish)
<i>MAP11</i>	Microtubule-associated protein 11	Cytokinesis Microtubule network stabilizer	MCPH25	AR	Non-sense (49)	Developmental delay, intellectual disability, ADHD, tethered spinal cord (49)		<i>map11</i> (mouse, zebrafish)
DNA DYNAMICS								
<i>MCPH1</i>	Microcephalin	Chromosome condensation Cell cycle checkpoint regulator DNA damage response	MCHP1	AR	Non-sense, deletion, frameshift, missense (non-conservative), splicing (3, 4, 6, 50–53)	Intellectual disability, growth retardation, cortical malformations (50, 54)	Pre-mature chromosome condensation syndrome (50, 54)	<i>W04A8.1</i> (<i>C. elegans</i>), <i>mcp1</i> (<i>Drosophila</i>), mouse, zebrafish)
<i>ZNF335</i>	Zinc finger protein 335	Transcriptional regulator	MCPH10	AR	Splicing, missense (non-conservative), frameshift (55–57)	Cortical malformations, facial malformations, seizures, hearing loss, motor problems, short stature, pre-mature death (55–57)		<i>CG8388</i> (<i>Drosophila</i>), <i>zfp335</i> (mouse), <i>znf335</i> (zebrafish)
<i>PHC1</i>	Polyhomeotic homolog 1	Chromatin remodeler	MCPH11	AR	Missense (non-conservative) (58)	Intellectual disability, short stature (58)		<i>phc1</i> (mouse, zebrafish)
<i>ANKLE2</i>	Ankyrin repeat and lem domain containing 2	Nuclear envelope disassembly	MCPH16	AR	Non-sense, missense (non-conservative) (4, 59)	Cortical malformations, facial dysmorphism, pigmentation abnormalities, motor problems, seizures, vision problems, anemia (4, 59)		<i>lem-4</i> (<i>C. elegans</i>), <i>ankle2</i> (<i>Drosophila</i>), mouse, zebrafish)

(Continued)

TABLE 1 | Continued

Gene name	Description	Cell process	Disease ID	Inheritance	Mode of inactivation	Other clinical features	Other associated conditions	Orthologs
<i>NCAPD2</i>	Non-SMC condensin I complex subunit D2	Chromosome condensation Sister chromatid disentanglement	MCPH21	AR	Splicing, missense (60, 61)	Intellectual disability, growth retardation, short stature (61)		<i>dpy-28</i> (<i>C. elegans</i>), <i>cap-d2</i> (<i>Drosophila</i>), <i>ncapd2</i> (mouse, zebrafish)
<i>NCAPD3</i>	Non-SMC condensin II complex subunit D3	Chromosome condensation Sister chromatid disentanglement	MCPH22	AR	Frameshift, splicing, missense (non-conservative) (61)	Short stature, limb hypertonia, seizures (61)		<i>hcp-6</i> (<i>C. elegans</i>), <i>cap-d3</i> (<i>Drosophila</i>), <i>ncapd3</i> (mouse, zebrafish)
<i>NCAPH</i>	Non-SMC condensin I complex subunit H	Chromosome condensation Sister chromatid disentanglement	MCPH23	AR	Missense (non-conservative) (61)	Intellectual disability (61)		<i>barren</i> (<i>Drosophila</i>), <i>ncaph</i> (mouse, zebrafish)
<i>NUP37</i>	Nucleoporin 37	Nuclear pore complex Kinetochore attachment	MCPH24	AR	Non-sense (61)	Intellectual disability, cortical malformations, clinodactyly (61)		<i>nup37</i> (<i>Drosophila</i> , mouse, zebrafish)
SIGNALING								
<i>CDK6</i>	Cyclin-dependent kinase 6	Cell cycle checkpoint regulator	MCPH12	AR	Missense (non-conservative) (62)	Facial dysmorphism, intellectual disability, cortical malformations (62)		<i>cdk-4</i> (<i>C. elegans</i>), <i>cdk6</i> (mouse, zebrafish)
<i>MFS2A</i>	Major facilitator superfamily domain-containing protein 2A	BBB lipid transporter Cell cycle checkpoint regulator	MCPH15	AR	Missense (non-conservative) (4, 63, 64)	Intellectual disability, motor problems, pre-mature death, seizures, cortical malformations (4, 63, 64)		<i>mfsd2a</i> (mouse), <i>mfsd2aa/mfsd2ab</i> (zebrafish)
<i>WDFY3</i>	WD repeat and FYVE domain containing 3	Wnt signaling	MCPH18	AD	Missense (non-conservative) (65)	Intellectual disability (65)		<i>wdfy-3</i> (<i>C. elegans</i>), <i>blue cheese</i> (<i>Drosophila</i>), <i>wdfy3</i> (mouse, zebrafish)
<i>COPB2</i>	Coatamer protein complex subunit beta 2	Cellular trafficking Cell cycle checkpoint regulator	MCPH19	AR	Missense (non-conservative) (66)	Developmental delay, low body weight, blindness, spasticity (66)		<i>E03H4.8/copb-2</i> (<i>C. elegans</i>), β' COP (<i>Drosophila</i>), <i>copb2</i> (mouse, zebrafish)

AR, autosomal recessive; AD, autosomal dominant.



role of each protein encoded by the human MCPH genes to illustrate this model, we also draw from studies of orthologous genes in the murine, nematode, and fly models, as required.

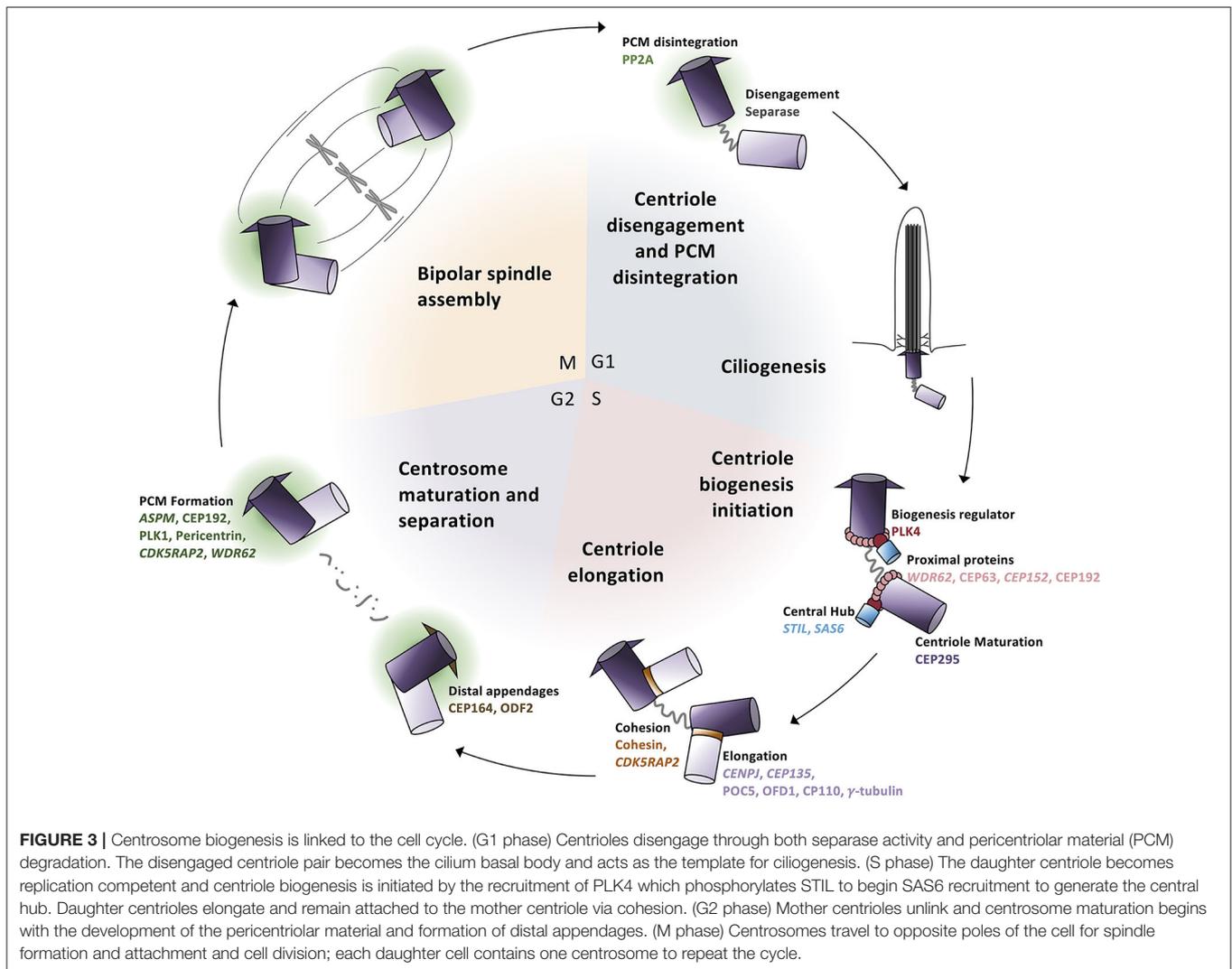
Centrosomes and the Cell Cycle

For many years, MCPH was considered a “centriolopathy” because most of the first genes implicated in causing the

disease were involved in centriole biogenesis. Centrosomes are essential for establishing the mitotic spindle during cell division and nucleating the ciliary axoneme during quiescence. Centrosomes are composed of a pair of centrioles (termed mother and daughter centrioles) and associated pericentriolar material (PCM). Centriole biogenesis is tightly linked to the cell cycle (Figure 3). In G1, just after cell division, the two centrioles from one centrosome are loosely linked together. In S phase, this link weakens further and is preceded by pro-centriole formation (i.e., new daughter centriole biogenesis). In G2, centrosome separation occurs and each new centrosome begins maturation. Finally, in mitosis, each centrosome travels to opposite sides of the cell during spindle formation in preparation for cell division. Therefore, there are four key processes essential in the centrosome cycle that are critical in terms of microcephaly: centriole biogenesis, centriole maturation, centriole tethering, and spindle formation. Variants in genes that are involved in these four key processes have recently been connected with MCPH (Table 1).

Centriole Biogenesis

To initiate the formation of daughter centrioles in G1–S phase, the original daughter centriole must first become duplication competent (i.e., must mature into a so-called “mother” centriole). Subsequently, a number of proteins are recruited to the proximal ends of the “grandmother” and “mother” centrioles (Figure 3). The scaffold protein WDR62 recruits CEP63 and CEP152, which form a ring-like structure at the mother centriole’s proximal end, and triggers the recruitment of PLK4, a polo-like kinase known



as the master regulator of centriole duplication, to the site of daughter centriole formation (75–79).

Following the recruitment of proteins to the mother centriole, PLK4 autophosphorylates itself concurrently with phosphorylating and recruiting STIL (80). PLK4 and STIL subsequently recruit SAS6 to form the template for the nascent daughter centriole (Figure 3). STIL and SAS6 oligomerize into a 9-fold symmetrical ring structure to form the unstable “cartwheel” central hub (80–82). Following central hub formation, proteins such as CENPJ and CEP135 are finally recruited, which aid in regulating and stabilizing central tube elongation and initiating singlet microtubules to assemble around the central hub (80, 83–87).

Centriole Maturation

The centrosome-associated pericentriolar material (PCM) is composed of a protein matrix and is responsible for anchoring and nucleating microtubules; just prior to mitosis, this matrix undergoes expansion. Master regulators of PCM maturation

recruit coiled-coil proteins as well as the attachment of γ -tubulin to the centrosome by CDK5RAP2; these activities ensure the formation of a PCM scaffold that permits centrosomal microtubule nucleation, which is an essential step in astral microtubule formation (Figure 3) (88–93).

Similar to the centrioles themselves, PCM assembly and disassembly is tightly linked to the cell cycle. During mitotic exit, the dense PCM must disassociate in order to drive centriolar separation (disengagement); daughter centriole biogenesis cannot occur if the so-called “grand-mother” and “mother” centrioles fail to separate because they are entrapped within the PCM (94, 95). Several centrosomal effector molecules therefore become dephosphorylated to promote their destabilization and result in a fragmented PCM, which allows centriole biogenesis to occur (96, 97).

Centriole Tethering

Following mitosis, the mother and daughter centriole become disengaged such that the tight cohesin fiber connections between

them loosen (95, 98). This requires PCM disintegration and separate protease activity. In G1, disengagement allows the mother centriole to become the basal body in order to nucleate the cilium but also licenses centriole biogenesis in S phase. The newly formed daughter centrioles are tightly bound (engaged) to the mother centriole via cohesin, which is maintained by CDK5RAP2 and functions to limit centriole biogenesis to once per cell cycle (99, 100). Finally, during mitosis, the two engaged centriole pairs (i.e., the centrosomes) lose their loose connection, become separated, and move to opposite sides of the cell where they begin mitotic spindle formation (Figure 3).

Spindle Formation

The mitotic spindle is responsible for the accurate segregation of chromosomes during cell division. The spindle depends heavily on dynamic microtubule activity for its function: astral microtubules connect the centrosome to the cell cortex, kinetochore microtubules connect the condensed chromosomes to the centrosome, and polar microtubules overlap at the central spindle to connect the two spindle poles (Figure 4). Each microtubule subtype performs specific tasks. Polar microtubules drive the separation of the two centrosomes, direct the positioning of the cleavage furrow, and promote abscission (Figure 4A). Astral microtubules serve to position the mitotic spindle and direct cleavage orientation (Figure 4B), while kinetochore microtubules are responsible for accurately segregating DNA to the opposite poles (Figure 4C). Predictably, many MCPH genes have roles in regulating spindle dynamics, including many of the genes that are involved in centriole biogenesis and PCM maturation (Figure 4D).

During anaphase, the highly abundant polar microtubules emanating from each of the spindle poles overlap centrally in order to form the stable central spindle (Figure 4). This structure is necessary to recruit proteins that position the cleavage furrow and trigger its contraction to form the midbody. Many of the centrosomal proteins that influence microtubule nucleation, such as ASPM, CDK5RAP2, and CIT, are recruited to the midbody during cytokinesis, highlighting the multifunctional roles of these proteins (101). In addition to these proteins, microtubule-associated proteins and their regulators also affect central spindle dynamics. CIT, which is a component of the contractile ring, recruits the microtubule-associated protein KIF14 to the central spindle, where it functions to stabilize the central spindle microtubule network and promote cytokinesis (Figure 4A) (102–105). MAP11 is similarly required at the midbody to promote abscission (49).

The astral microtubules in particular are essential for the orientation of the mitotic spindle (and thus cell division orientation). Rotation of the mitotic spindle depends on forces generated between the cell cortex and the astral microtubules. Minus-end-directed activity of a dynein–dynactin complex, coupled with the cortical anchoring of the astral microtubules, generates a pulling force which allows for rotation and positioning of the spindle (106–108). ASPM seems to be a major player in this process since it binds to nuclear mitotic apparatus (NuMA), which localizes to the spindle poles and the cortex, and subsequently recruits the dynein–dynactin complex to the

spindle poles, where it acts as the force generator in spindle positioning (Figure 4B) (109–112).

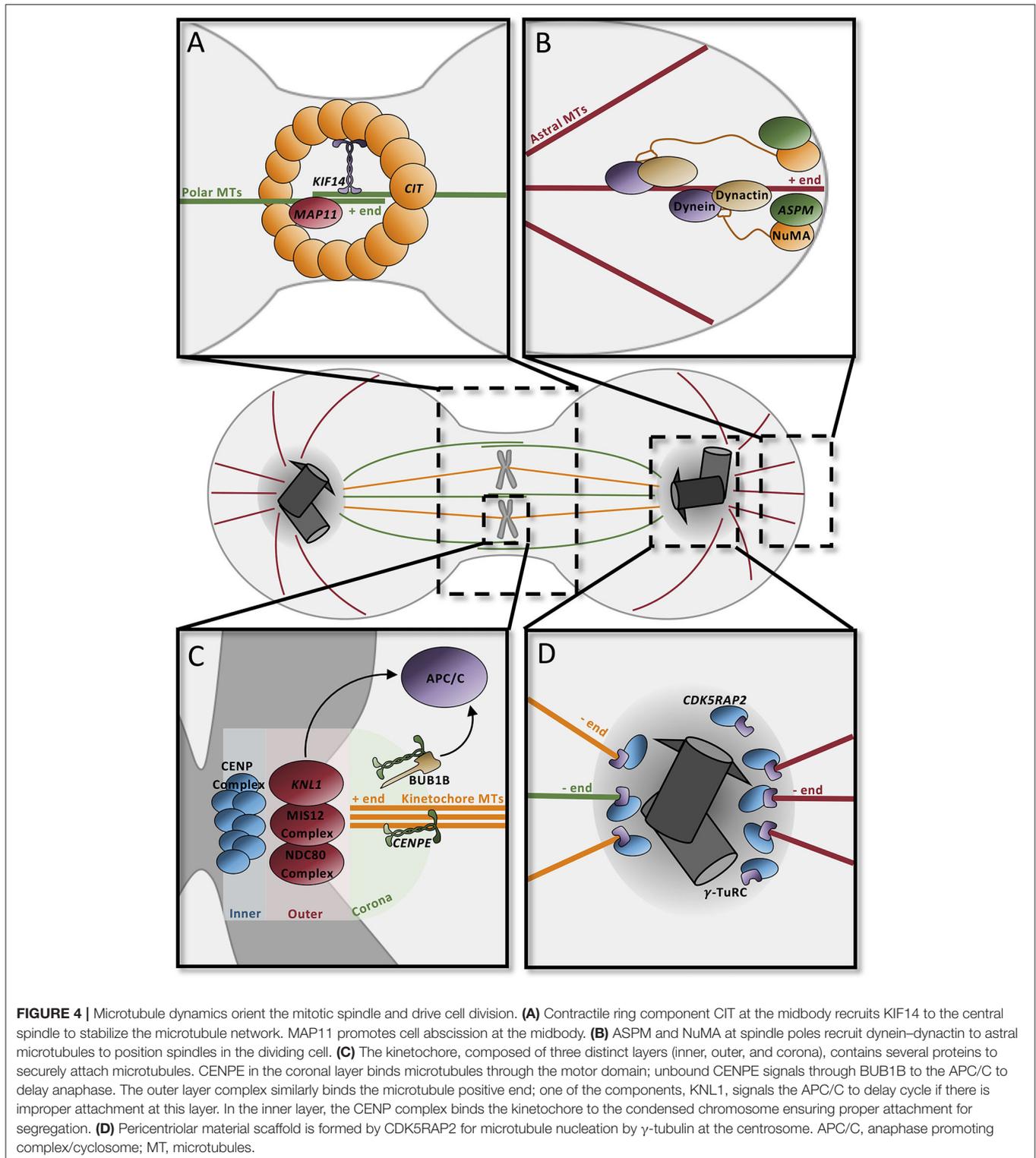
The kinetochore microtubules are required for the faithful segregation of the chromosomes during cell division; aberrant segregation can lead to chromosomal instabilities or aneuploidy, which can be toxic to the cell. Under the “search and capture model,” microtubules emanating from the spindle poles seek out and attach to the heavily scaffolded kinetochore. The multilayered kinetochore is largely responsible for ensuring this correct attachment and for triggering cellular alarms when microtubules are not appropriately attached (Figure 4D). One of the outermost microtubule-capturing kinetochore components is the centromere-associated protein E (CENPE), which is a large, kinesin-like motor protein that binds to microtubules through its motor domain (113, 114). KNL1, which is located in a complex more interiorly in the kinetochore, is similarly required for microtubule binding (115). If microtubules are incorrectly attached to the kinetochore, then the spindle assembly checkpoint (SAC) will be activated, delaying mitosis until attachment has been corrected. For instance, microtubule-unbound CENPE binds to BUB1B and triggers its phosphorylation which leads to a “delay anaphase” signaling cascade that culminates on the anaphase-promoting complex/cyclosome (APC/C) (114, 116). Similarly, the KNL1 complex functions redundantly with CENPE to delay the cell cycle (117).

DNA Dynamics During the Cell Cycle

Similar to centrosomes, DNA dynamics are tightly linked to the cell cycle. At the onset of mitosis, one of the cell cycle checkpoints ensures that chromosomes have been accurately condensed and that breakdown of the nuclear envelope occurs; together, these permit the proper pairing of homologous chromosomes at the metaphase plate and the subsequent segregation of sister chromatids into the presumptive daughter cells during anaphase. Following mitosis, cells in G1 become transcriptionally active. Throughout the cell cycle, DNA is continually monitored for damage, which must be repaired in order for the cell to continue to progress through the cycle. Dysregulation of DNA dynamics is thus capable of creating delays in the cell cycle and affecting the overall trajectory of a cell within a developing system, such as the brain. Specifically, delays in the cell cycle of neural progenitors will reduce the proliferative pool and the subsequent number of differentiated neurons within the developing brain. Therefore, because of the tight link between DNA dynamics and cell cycle timing, variants in genes that affect chromosomal condensation, transcriptional regulation, DNA damage responses, and nuclear envelope breakdown are increasingly being implicated in causing MCPH (Table 1).

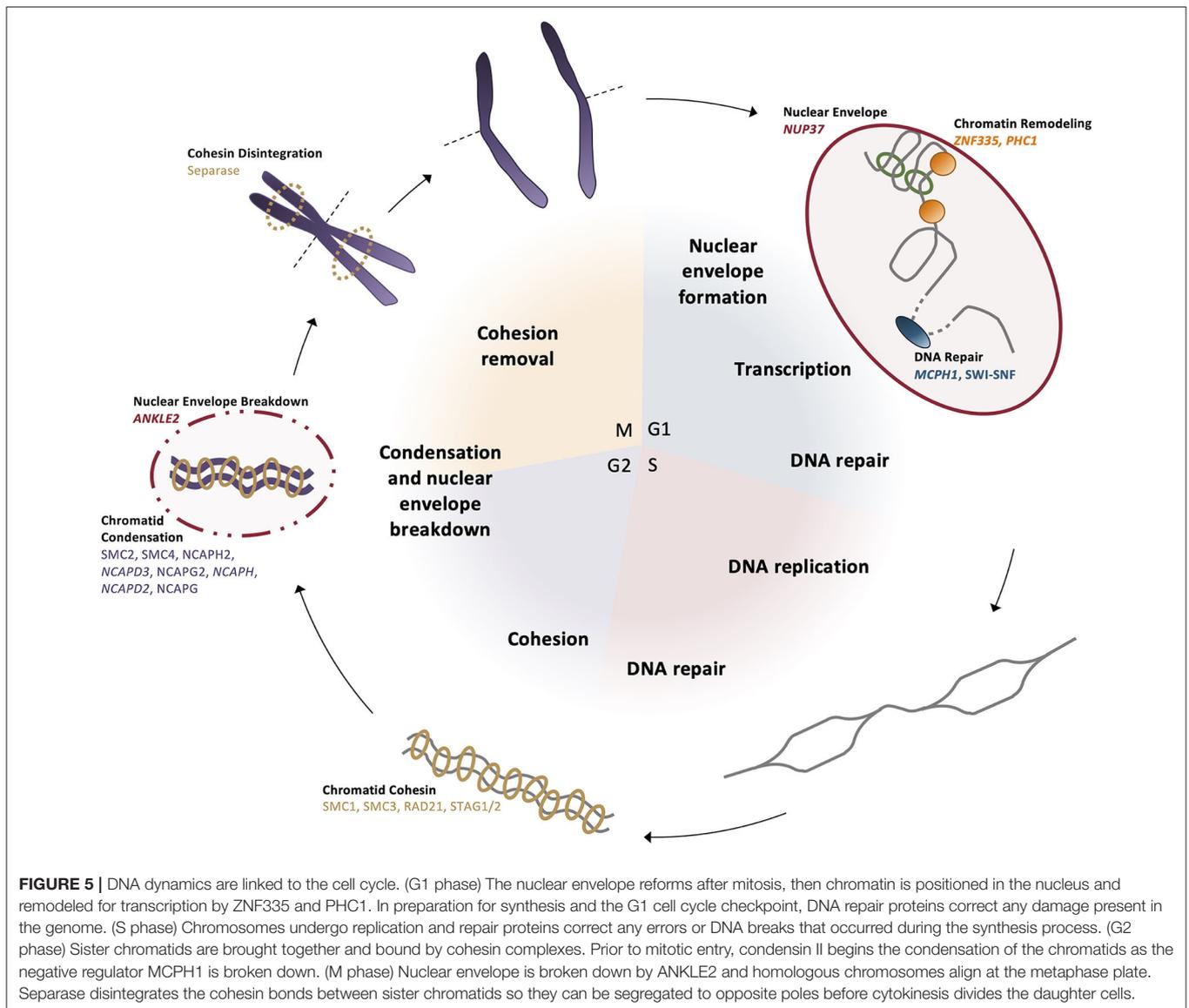
Condensation and Transcriptional Regulation

Chromosomal condensation is one mode by which transcription is regulated across cell types and throughout the cell cycle. The shift between heterochromatin and euchromatin regulates the genes that are accessible to transcriptional machinery, shifting the transcript profile of each cell. One MCPH gene, *ZNF335*, is an H3K4 methyltransferase that binds to



heterochromatin upstream of the neuron-restrictive silencer factor (NRSF) locus in neural stem cells to prevent differentiation pathways; this brain-specific expression pattern is turned off during the dynamic remodeling in the transition to differentiation (**Figure 5**) (55). PHC1 also represses transcription

through chromatin remodeling by ubiquitinating histone H2A to maintain condensation of specific genomic regions (58). Since the switch from proliferation to differentiation requires changes in gene regulation and expression, dynamic chromatin remodeling permits transcriptional machinery binding to



various genomic regions related to cell cycle progression and differentiation.

There are many nuclear proteins involved in reducing the length of the chromosomes, some of which are causative in primary microcephaly (118, 119). The primary complexes acting to further condense the chromosomes both laterally and axially are condensin I and condensin II, which share the same structural maintenance core (SMC) subunits but differ in their associated NCAP family subunits (Figure 5). In preparation for entry into mitosis, the SMC associates with NCAPH2, NCAPD3, and NCAPG2 to form condensin II in the nucleus, which localizes to the chromosomes and creates an axially rigid structure of interacting chromosome regions, shortening the overall length of the chromosomes (119, 120). Condensin II localization to the chromosomes is restricted to this phase, as throughout the rest of the cell cycle, it is negatively regulated by MCPH1 until condensation can be coupled with centriole duplication, ensuring

that all necessary mitotic structures are formed by the end of G2 (50, 121). Lateral compaction occurs after nuclear envelope breakdown when the condensin I complex—including NCAPH and NCAPD2—can associate with the chromosomes to loop the chromatin around the established axial patterning created by condensin II (Figure 5) (122).

Beginning in S phase, the newly replicated sister chromatids must be connected through to the early mitotic stages, until finally at anaphase, sister chromatids are separated. Sister chromatid cohesion is mediated by the cohesin complexes, which also contain SMC molecules but differ in their accessory protein subunits (Figure 5). This ring-like complex encircles each of the sister chromatid pairs to ensure they are tethered together. At anaphase, the complex is degraded by the protease separase, which is the enzyme that facilitates centriolar disengagement, linking DNA dynamics and centrosome function through the cell cycle (123). This ensures that the sister chromatids are

faithfully segregated, and each daughter cell contains identical DNA content. In the event that the cohesin complex prematurely dissociates, kinetochore-centromeric attachments fail to occur and genomic instability can result.

DNA Damage Response

Prior to synthesis and mitosis, the DNA damage response ensures the accurate passage of genomic information to both daughter cells; if the genome is damaged beyond a repairable threshold, the cell is instead sent into the apoptotic pathway to prevent aberrant cell function or growth and to maintain a healthy cell population. MCPH1 localizes to chromatin that has been damaged by ionizing radiation and prevents compaction of the chromatin at that locus by inhibiting the condensin II complex and, thus, mitotic onset until the repair machinery is able to correct the sequence (Figure 5) (124–126).

Nuclear Envelope

In interphase, the nuclear envelope is critical for the movement of proteins into and out of the nucleus, regulating transcription factor access to the genome and the proteins required for the transition to differentiation. NUP37 is a microcephaly-associated nucleoporin that encodes an essential component of the nuclear pore complex (NPC) (Figure 5). It is a component of the ring-shaped Y-complex on the surface of the nuclear envelope, creating much of the structure for the nuclear pore (127). The presence of the Y-complex is necessary for nuclear pore stability and continued proliferation (61). However, during mitosis, the nuclear envelope must disintegrate in order to permit the segregation of the condensed chromosomes. While cells are in interphase, chromatin is bound to the nuclear envelope to maintain each chromosome in its defined region of the nucleus. In preparation for the breakdown of the nuclear envelope during mitosis, ANKLE2 phosphorylates envelope components to reduce the binding affinity between chromatin and the nuclear envelope (128). Once this interaction is broken, the nuclear envelope can begin to disassemble; upon mitotic exit, ANKLE2 dephosphorylates components so the nuclear envelope can reform and reestablish its interaction with the chromatin within the nucleus (128).

Signaling

The decision for a cell to progress through the cell cycle and divide relies upon numerous signals. For a cell to enter S phase, the cell must assess DNA integrity, metabolic state, and developmental cues—providing these all satisfy certain thresholds, the cell may progress through the cell cycle, which is itself regulated by a series of checkpoints that require the cyclic activity of positive regulators [cyclins and cyclin-dependent kinases (CDKs)] and negative regulators (examples include p53, p21, and retinoblastoma protein). Here, we discuss MCPH genes that influence signaling pathways, such as those involved in cell cycle checkpoints, metabolism, and Wnt signaling.

Cell Cycle Regulators

One MCPH gene, *CDK6*, directly regulates the cell cycle; it is responsible for the progression of the cell through G1 phase

and the G1/S phase transition (62, 129). In addition, there are two MCPH genes, *COPB2* and *MFSD2A*, that more indirectly affect the cell cycle (66, 130, 131). The first, *COPB2*, is typically associated with trafficking between the Golgi apparatus and the endoplasmic reticulum, but it has recently been shown to regulate several cell cycle proteins (132). Knockdown of *COPB2* increases the expression of CDK inhibitors (P16 and P21) and decreases the expression of cyclin A1 and A2, which are responsible for progression through S phase. *MFSD2A* encodes a fatty acid transporter that acts at the blood–brain barrier (133, 134). It is responsible for the uptake of lysophosphatidylcholines (LPCs), such as those derived from docosahexanoic acid (DHA), which are not synthesized within the brain but are essential for neurogenesis. Specifically, in the early stages of neural stem cell differentiation, DHA promotes cell cycle exit and subsequent differentiation by decreasing the expression of several key cyclins, thus preventing the transition from G1 to S phase (130, 131).

Metabolism

MFSD2A also performs a metabolic role. DHA is a major component of the brain lipid profile as it comprises many of the phospholipids within the brain. Mutations in *MFSD2A* result in increased plasma levels of LPCs and a corresponding decrease in LPC uptake into the brain; this reduced uptake is associated with both lethal and non-lethal microcephaly in humans and animal models (63, 64, 133, 135). The presence of DHA in the brain suppresses the activity of master transcriptional regulators of sterol and fatty acid synthesis (133). Accordingly, reduced uptake of DHA due to mutations in *MFSD2A* results in the increased expression of the master transcriptional regulators and their downstream targets. This indicates that the lipid metabolic pathways must be tightly regulated during neurogenesis, and is highlighted by the link between DHA levels within the brain and proliferation (136).

Wnt Signaling

Canonical Wnt signaling activation, at its core, involves the binding of a Wnt-protein ligand to a Frizzled family receptor, which in turn activates the intracellular messenger protein, Disheveled, to influence gene transcription via β -catenin accumulation. Canonical Wnt signaling promotes proliferative cell divisions (i.e., the production of progenitor cells), whereas its loss leads to neuronal differentiation (137–140). *ASPM* appears to act as a positive regulator of Wnt signaling by preventing the proteasome-mediated degradation of Disheveled (141–143). Conversely, *WDFY3* attenuates Wnt signaling; it is responsible for degrading Disheveled aggregates, thus reducing β -catenin levels (65).

FACILITATING PRIMARY MICROCEPHALY DIAGNOSIS

As summarized, MCPH genes are involved in a number of cellular processes, such as centriole biogenesis, mitotic spindle formation, transcription, DNA damage responses, and signaling. Obviously, screening for the 25 known genes in MCPH patients

via gene panels or directed sequencing is the simplest method of diagnosing patients and is likely to be successful in about 50% of patients, as previously found (144). However, with advances in WGS, how do we address the remaining 50% of patients? In this section of the review, we discuss a range of aspects to consider when prioritizing variants in patients.

Linking Cell Biology to Disease

A thorough understanding of the etiology of a disease can guide the diagnostic process. Thus far, we have outlined the molecular roles of each of the MCPH genes known to date, and although each of the thus far implicated genes appear to be involved in distinct cellular processes, MCPH mutations commonly affect the cell cycle exit fraction by pre-maturely increasing the ratio of differentiating to proliferating cells or by triggering apoptosis. For instance, incorrectly positioned mitotic spindles promote asymmetric divisions, thereby increasing the cell cycle exit ratio and cell division delays mimic differentiating cells which have exited the cell cycle. We would therefore predict that mutations in functionally related genes would be likely implicated in the disease. In support of this, a *FZR1* variant, which encodes a component of the APC/C complex and is responsible for driving the cell cycle, has recently been identified in a patient with MCPH-like phenotypes (145). Similarly, a patient with a mutation in *TUBGCP5*, which is a centrosomal component that affects microtubule nucleation and spindle orientation, presents with MCPH (146). Finally, a *METTL5* variant has also recently been identified in a patient with clinical features matching that of MCPH, highlighting the role of epigenetics and transcriptional regulation in the etiology of MCPH (147). Although these three genes are not listed in OMIM as of August 2020 as MCPH genes, we expect that these will be included following database updates and identification of additional patients with mutations in these genes.

Translation From Genetic Models

One issue when diagnosing patients is that many genes simply do not have a recognized molecular or cellular function ascribed to them. However, the study of these genes in model organisms may reveal a function related to processes disrupted in microcephaly. Not only do most of the MCPH genes have conserved functions across the animal kingdom (Table 1), but mutations in these genes also show a conserved set of phenotypes in model organisms (phenologs); although these phenotypes do not always perfectly mimic the human disease condition, they do reflect perturbation of a common pathway, which gives us a better understanding of whether a given gene is likely to be causal in disease. For instance, modeling MCPH gene mutations in zebrafish, mice, and *Drosophila* typically results in animals with small heads, whereas in *Caenorhabditis elegans*, embryonic lethality with abnormal cell division phenotypes is often observed (49, 65, 69, 80, 83, 148–155). In this way, we can not only validate potential candidates but also begin to screen for possible new candidates that may subsequently be found in human patients. Loss of *cntrob* in zebrafish or *Hmgn2* in mice results in microcephaly, although, as of yet, they are not associated with microcephaly in humans (156, 157). Similarly, pioneering work

in *C. elegans* has revealed the importance of SPD-2 (CEP192 in humans) in centriole duplication and PCM maturation, but despite the clear association of other core centriole biogenesis components with microcephaly, CEP192 has not yet been implicated (80, 158). While these genes are not yet associated with MCPH, this may be due to the rarity of these variants in human populations or highly deleterious effects resulting in lethality. Additionally, the phenotypes seen in model organisms may be more profound in the lab environment than they would present in humans, decreasing their chances of being discovered.

Analyzing Inheritance Patterns

Another mechanism of prioritizing variants is to examine the inheritance pattern. With the exception of *WDFY3*, all MCPH variants are autosomal recessively inherited (Table 1). Many microcephaly patients exhibit homozygous variants in the disease-causing gene due to consanguinity, but increasingly, compound heterozygous variants are being discovered. Therefore, in non-consanguineous families, performing triad sequencing and using filtering methods that include compound heterozygous variants are essential. However, recent evidence suggests that the inheritance of MCPH may be more complicated than originally thought and alternative forms of inheritance should be considered in difficult-to-diagnose patients. For instance, similar to *WDFY3*, mutations in *DPP6*, which encodes a dipeptidyl peptidase protein, cause autosomal dominant microcephaly in addition to intellectual disabilities, indicating that it may also be necessary to screen through heterozygous variants in patients (159). Furthermore, there is increasing evidence that MCPH may occasionally follow an oligogenic inheritance pattern; a recent study found that microcephaly patients have a higher mutational burden in genes implicated in causing MCPH (69). Additionally, the digenic inheritance of *CDK5RAP2* and *CEP152* heterozygous mutations causes Seckel syndrome, a disease which includes microcephaly as a clinical feature (24). Again, turning to genetic models may validate instances where digenic inheritance is predicted to be necessary for phenotypic presentation or where modifying genes affect phenotypic severity. In the fish model, for example, homozygous mutations in both *aspm* and *wdr62* are necessary to produce the microcephaly phenotype (69). Considering that ciliopathies are well-established digenic conditions, and like microcephalies, depend upon proper cell cycle timing and centrosome function (Figure 3), it is not challenging to envision a model in which these diseases genetically parallel each other. Conversely, a mutation in *Ttc21b* causes genetic background-dependent microcephaly in mouse; quantitative trait locus analysis revealed a missense mutation in one genetic background but not the other that was able to enhance the *Ttc21b* neural phenotypes (160). Therefore, clinicians may have to look past traditional autosomal recessively inherited variants in a single gene in particularly challenging to diagnose patients.

Determining Mode of Gene Inactivation

Intriguingly, most of the MCPH-causing mutations are non-sense, frameshift, or splicing mutations, all of which typically result in a complete loss-of-function of the protein product

(**Table 1**). However, this also implies that other types of nullifying mutations, such as structural variants, copy number variants (CNVs), or non-coding regulatory variants, may be causative in microcephaly. Already, larger deletion mutations in *MCPH1* and *ASPM* have been identified in MCPH patients, in addition to a translocation break in *ASPM* that disrupts the coding sequence (3, 36, 37, 51). However, these types of variations are likely to be increasingly discovered as our methods of identifying these variants improve. Similarly, the use of transcriptomics, such as RNA-seq, may expedite our identification of non-coding variants in patients; the use of transcriptomics in a patient with microcephaly-micromelia syndrome found a non-coding mutation in *DONSON* that caused aberrant splicing (161). Multimethod approaches to identifying causal mutations may therefore be necessary, in addition to using tools that are capable of detecting a wide range of variants.

Evolution of Brain Size in Primates

One of the hallmarks of human evolution is an increase in brain size and complexity, a change that was accompanied by increased cognitive power. Interestingly, there is a correlation between genes predicted to be responsible for driving the increase in human brain size and those that are implicated in causing MCPH. For instance, *MCPH1*, *CDK5RAP2*, *CENPJ*, and *ASPM* have been shown to have undergone positive selection during primate evolution (162–165). Researchers speculate that over the course of evolution, MCPH genes may have accumulated genetic changes that permitted the increase in hominid brain size—specifically, these changes likely altered the rate of cell division in proliferating neuronal stem cells. Conversely, the disease state may be an example of atavism, in which nullifying mutations recreate a more ancestral state. Another possibility is the changes in copy number may underlie hominid brain size changes; a recently discovered example is *NOTCH2NL*, in which three copies are necessary for normal human development (166). The duplication or deletion of the locus in which *NOTCH2NL* is located results in macrocephaly or microcephaly, respectively. Therefore, evolutionary analyses, such as those determining whether a gene undergoes positive selection through the course of hominid brain evolution, may be one more mechanism by which microcephaly-causing variants are prioritized [reviewed in Gilbert et al. (166)].

REMAINING QUESTIONS

Why do mutations in MCPH genes specifically affect neurogenesis? While some MCPH genes have enriched expression in neural progenitors or have a biological function limited to the brain (e.g., *MFSD2A*), many genes are ubiquitously expressed and have biological functions that are necessary in many tissue types (18, 134). Therefore, a major question is why mutations in the MCPH genes, which are typically nullifying mutations, specifically cause microcephaly, with generally few abnormalities outside of the head and brain. This is especially true when we consider that in the *C. elegans* model, mutations in orthologous MCPH genes result in lethality, which would be predicted based on the essential function of these genes in cell division. One similarity between neuroprogenitors and *C. elegans* development though

is that the cell cycle length is remarkably short; in humans, the length is shorter in comparison to other cell types, and in *C. elegans*, the first few cell divisions occur extremely rapidly and are stereotypically oriented (167, 168). In fact, G1 length has been shown to be an essential regulator of the switch between proliferation and differentiation, which explains why mutations in MCPH genes, which generally cause a lengthening in the cell cycle and thus pre-maturely increase the cell cycle exit fraction, have dramatic effects on the number of progenitors remaining in a proliferative state (129, 169). The brain therefore appears to be more sensitive to changes in cell cycle length relative to other tissue types, an effect that may be appropriately modeled using the *C. elegans* genetic system.

Another possibility may lie in alternative splicing. The brain undergoes the most alternative splicing events than any other tissue and expresses the largest number of splicing factor genes (170). This means that the brain produces more diverse protein isoforms than other tissue types. Accordingly, there are many examples of splicing mutations in MCPH genes (**Table 1**); it is possible that the disruption in splicing in these genes affects the protein isoforms that are uniquely expressed in the brain. For instance, a splicing mutation in *KNL1* specifically affects neuronal progenitor cells, but fibroblasts and neural crest cells expressing the same mutation are unaffected (171). Although not every MCPH mutation affects splicing, this may be one mechanism that contributes to the brain-specific presentation.

Finally, the classical clinical definition of MCPH describes an isolated disorder affecting the head and brain size, with typically no other malformations. However, as more patients continue to be discovered and diagnosed with MCPH, the clinical landscape of primary microcephaly expands. We now know that in addition to a small head, MCPH is frequently associated with cortical and facial malformations, intellectual disabilities, and seizures, in addition to short stature, heart problems, and in extreme cases, pre-mature death (**Table 1**). Many of the genes are also implicated in causing different but related diseases, such as Seckel syndrome or microcephalic primordial dwarfism, indicating that there is likely an overlapping pathophysiology between these conditions, and they may represent a disease spectrum rather than individual conditions. In support of this, many genes that are implicated in causing syndromic microcephaly affect the same pathways as MCPH genes, indicating that cell cycle dysregulation underlies both isolated and syndromic microcephalies. This reflects the prediction that mutations in genes affecting cell cycle progression would present with more pleiotropic effects.

CONCLUDING REMARKS

MCPH is a heterogeneous disorder that, like many rare diseases, has been challenging to diagnose despite advances in genomics. Therefore, there is a need to understand the genetics and cell biology underlying the disease in order to expedite discovery and genetic diagnosis. This review has shown that microcephaly is caused by aberrant cell cycle regulation by summarizing the molecular functions of each of the known MCPH-causing genes and their associated cellular phenotypes, as well as providing examples of novel MCPH candidates.

In addition to furthering our understanding of the disease pathogenesis, we have also provided insights on the genetics and inheritance of MCPH with the hopes of facilitating the variant prioritization process in patients with microcephaly. Altogether, the incorporation of each of these disease facets in the process of identifying the MCPH-causing gene(s) will improve diagnosis rates in these patients and will guide family planning and personalized treatments but also forms a template for the inclusion of multidisciplinary approaches in the diagnostic process.

AUTHOR CONTRIBUTIONS

FJ and MT-G: conceptualization and funding acquisition. FJ and AS: writing—original draft preparation. MT-G: writing—review

and editing and supervision. All authors have read and agreed to the final manuscript version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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