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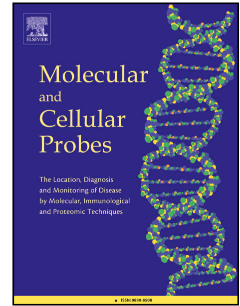

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CEP63

What Next-Generation Sequencing (NGS) technology has enabled us to learn about Primary Autosomal Recessive Microcephaly (MCPH)

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Abstract

The impact that next-generation sequencing technology (NGS) is having on many aspects of molecular and cell biology, is becoming increasingly apparent. One of the the most noticeable outcomes of the new technology in human genetics, has been the accelerated rate of identification of disease-causing genes. Especially for rare, heterogeneous disorders, such as autosomal recessive primary microcephaly (MCPH), the handful of genes previously known to harbour disease-causing mutations, has grown at an unprecedented rate within a few years. Knowledge of new genes mutated in MCPH over the last four years has contributed to our understanding of the disorder at both the clinical and cellular levels. The functions of MCPH proteins such as WDR62, CASC5, PHC1, CDK6, CENP-E, CENP-F, CEP63, ZNF335, PLK4 and TUBGPC, have been added to the complex network of critical cellular processes known to be involved in brain growth and size. In addition to the importance of mitotic spindle assembly and structure, centrosome and centriole function and DNA repair and damage response, new mechanisms involving kinetochore-associated proteins and chromatin remodelling complexes have been elucidated. Two of the major contributions to our clinical knowledge are the realisation that primary microcephaly caused by mutations in genes at the MCPH loci is seldom an isolated clinical feature and is often accompanied either by additional cortical malformations or primordial dwarfism. Gene-phenotype correlations are being revisited, with a new dimension of locus heterogeneity and phenotypic variablity being revealed.

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1. Introduction

The enormous impact that next-generation sequencing technology (NGS) is having on many aspects of molecular and cell biology, is becoming increasingly apparent. One of the most noticeable outcomes of the new technology in human genetics, has been the accelerated rate of identification of disease-causing genes since the first publication of disease gene identification using whole exome sequencing (WES) [1]. Especially for rare, heterogeneous disorders, the handful of genes previously known to harbour disease-causing mutations, has grown at an unprecedented rate within a few years. The field of neurodevelopmental genetics has been prominent in reaping the benefits of these developments [2] [3] and primary microcephaly is no exception. The identification of the genetic defects causing profound developmental abnormalities not only provides unique insight into the underlying developmental and cellular processes, but also enables the development of molecular genetic tests to improve diagnosis and facilitate family planning. There have been many excellent reviews on both primary and postnatal microcephaly over the last five years.[4-9] The aim of this review is to focus on the most recent findings on autosomal recessive primary microcephaly (MCPH) and in particular to highlight how the new sequencing technologies have expedited the search for new genes. Knowledge of these genes and the proteins they encode has enabled the complementation of known pathways with new components and the linking of cellular mechanisms, thus painting a more complete picture of the molecular and cellular basis of MCPH.

Microcephaly is a clinical term for a “smaller than normal head”. It may develop prenatally or postnatally and may have a genetic or non-genetic cause. Microcephaly accounts for a significant fraction of childhood intellectual disability, autism and epilepsy and occurs both in isolation and as part of a broad range of neurodevelopmental syndromes with or without other cortical malformations; and with or without growth retardation. Primary autosomal recessive microcephaly (MCPH) is a developmental disorder that is characterized by prenatal onset of abnormal brain growth, resulting in an occipitofrontal head circumference (OFC) at birth which is at least two to

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three standard deviations below the mean for sex-, age and ethnicity. Patients have a characteristic head shape with sloping forehead [7, 10]. There are now 13 MCPH loci implicated in MCPH (Table 1), with mutations in the *ASPM* gene (MCPH5) accounting for 25-50% of cases [11-15], followed in frequency by mutations in *WDR62* [16-18].

2. Next-generation sequencing (NGS) in patients with MCPH

The mapping of disease loci in putative Mendelian forms of primary microcephaly, as in the case of many other rare genetic diseases, had been hindered by marked locus heterogeneity, small family sizes and diagnostic classifications that may not reflect the molecular pathogenesis. NGS technologies have relieved many of the these constraints by enabling the analysis of a large number of genes in a single assay, either in the form of a gene panel [19], the whole exome (WES) encompassing the coding and intronic flanking sequence of all known genes, or the whole genome (WGS)[20-22]. Although optimally performed in more than one individual, WES can be successful in identifying the disease-causing mutation(s) and gene in a single patient. Subsequent confirmation of the gene-phenotype association can be performed via the screening of additional patients. By 2010, there were seven known loci for primary microcephaly, labelled MCPH1 to 7, but only five known genes (*Microcephalin*/MCPH1, *CDK5RAP2*/MCPH3, *ASPM*/MCPH5, *CENPJ*/MCPH6 and *STIL*/MCPH7). The MCPH numbering has since been adjusted to accommodate newly-identified genes, of which there are now at least 13 (Table 1).

Contributory to the success in identification of new disease genes for primary microcephaly has been fact that all the phenotypes described to date show an autosomal recessive inheritance pattern. In many cases it has been possible to refine the candidate genomic region to parts of one or more chromosomes, either via whole genome linkage analysis or homozygosity mapping in large, often consanguineous, families. Following up with WES in a single affected family member, with the

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possible addition of an unaffected family member, has in many cases proved sufficient and successful in identifying the disease-causing mutations and disease gene. One of the greatest challenges of working with data generated by WES or WGS is the process of variant assessment, in order to prioritize variants for their potentially deleterious effects. This process involves filtering thousands of DNA variants, according to a process of elimination based on allele frequency, algorithms relating to possible functional effects at the protein level and evolutionary conservation of base pair changes, in order to eventually arrive at a single candidate disease-causing mutation for the disorder at hand. This process of filtering is substantially reduced under the hypothesis of autosomal recessive inheritance, most likely due to homozygosity for a single rare mutation or compound heterozygosity for two rare likely pathogenic mutations in the same gene in a single patient. The confirmation that each of the patient's parents is heterozygous for one of the mutations, adds another piece of evidence towards the certainty that the mutations found are likely to be the disease-causing mutations in the patient. This has been clearly demonstrated by the success of disease gene identification using NGS not only in primary microcephaly, but also in other genetically heterogenous predominantly autosomal recessive disorders, such as primary ciliary dyskinesia [23]. The NGS approach has also exposed the unexpected constellation of compound heterozygosity despite consanguinity, that may have been missed when applying only homozygosity mapping and Sanger sequencing [24].

3. Recurring themes: centrosomes, centrioles, microtubule aberrations, cell cycle dynamics and DNA damage response

The known MCPH genes encode proteins that are almost universally involved in the biology of centrioles, cellular organelles that are part of the centrosome and play critical roles in cell division and cell cycle checkpoints, thus highlighting the role of the centrosome and cell cycle dynamics in the aetiology of microcephaly (Figure 1). The assembly of a bipolar, microtubule spindle during

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mitosis is essential for accurate chromosome segregation and in animal cells, spindle formation is organized by centrosomes. Centrioles need to be duplicated exactly once every cell division cycle, in coordination with DNA replication, in order to maintain genome stability [25]. Some of the MCPH proteins are core centriolar components, e.g. CENPJ, STIL and CEP135, others have a nuclear or cytoplasmic localization during interphase and only locate to the spindle poles during mitosis e.g. WDR62, ASPM and CDK6 [18, 26, 27] and yet others interact with known centrosomal proteins, e.g. CASC5 and PHC1. This raises the question as to how mutations in genes encoding centrosomal and spindle pole-associated proteins lead to microcephaly and/or growth retardation.

During development of the mammalian cerebral cortex, neurons are generated from two principal classes of neural progenitor cells (NPCs), namely apical precursors (AP) which are somatic stem cell-like neuroepithelial cells and radial glial cells, and basal progenitors (BP) [28]. With the generation of neurons, the neuroepithelium transforms into a tissue with numerous cell layers, with the layer lining the ventricle, known as the ventricular zone being the most apical cell layer that contains most of the progenitor cell bodies [29]. AP cells exhibit apical–basal polarity, undergo mitosis at the ventricular (apical) surface and their cell bodies constitute the ventricular zone (VZ) [29-31].With the switch to neurogenesis, neuroepithelial cells downregulate certain epithelial features, and give rise to radial glial cells, which exhibit residual neuroepithelial as well as astroglial properties [32-34]. Radial glial cells represent more fate-restricted progenitors than neuroepithelial cells and successively replace the latter [29, 35]. BP (or intermediate NPCs) originate from apical mitoses, translocate their cell bodies through the VZ in the basal direction, form the subventricular zone (SVZ), downregulate apical–basal polarity (at least in rodents) and undergo mitosis in the basal VZ or SVZ [35, 36]. The balance between NPCs and neurons is dependent upon three principal types of AP and BP cell divisions: self-expanding symmetric proliferative, self-renewing asymmetric BP- or neuron-generating, and self- consuming neurogenic divisions. The switch of neural stem and progenitor cells from proliferation to differentiation during development is apparently a crucial determinant of brain size [30, 37]; and as to be expected there are a multitude of factors that play a

role in this highly complex process. One of the key determinants in the type of NPC division is proposed to be the orientation of the mitotic spindle. This determines the orientation of the cleavage plane and therefore the position of the two daughter cells [38]. Spindle orientation helps determine the neurogenic outcome of asymmetric progenitor divisions, thereby influencing neuron output and cerebral cortical expansion.

Compared to other cell types, cell cycle length can be remarkably short in developing neuroprogenitors [39]. Since these cells need to undergo rapid and temporally restricted expansion, efficient DNA replication is fundamental to ensure normal neural development. Concomitant with the progression of neurogenesis, cell-cycle length of cortical NPCs in the VZ is known to increase, and there are intriguing links between NPC cell-cycle length and neuron output [40]. Arai et al. [28] have elegantly shown that there are differences in cell cycle length between APs and BPs in the G1 and S phases of the cell cycle, and that BPs exhibit a substantially longer S-phase than APs, suggesting a greater time investment in quality control of replicated DNA in expanding NPCs [28].

The reduced volume of the cerebral cortex in primary microcephaly is suggested to result from aberrant neurogenesis, influenced by all of the above processes including cell division, DNA replication and genome maintenance. From the proteins so far identified as being affected by mutations in the MCPH genes, there is evidence for all of these processes being disrupted in one form or another. Mitotic defects, such as aberrant spindle pole positioning has been demonstrated for ASPM after siRNA-mediated knockdown of *ASPM* in non-neural human cells [41], in Drosophila *asp* mutants and after similar knockdown of *Aspm* in mice [42]. Strong support for spindle misalignment and disrupted symmetric vs asymmetric cell divisions has also been provided by Mcph1-deficient mice in which premature switching of neuroprogenitors from symmetric to asymmetric division was observed [43]. Mcph1 deficiency abrogated the recruitment of checkpoint kinase 1 (Chk1) to centrosomes, resulting in premature cyclin-dependent kinase 1 (Cdk1) activation.

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This in turn resulted in early mitotic entry and the uncoupling of mitosis from the centrosome cycle [44]. MCPH1 patient cells show premature chromosome condensation [45] and abnormal chromosome alignment has been observed in a mouse MCPH1 model [44]. Moreover, similar to ATR–Seckel syndrome cells, *MCPH1*-mutant cell lines show defective G2–M checkpoint arrest, nuclear fragmentation and supernumerary mitotic centrosomes after DNA damage, thus suggesting a role for MCPH1 in the ATR DNA damage response [43].

4. New genes, new phenotypes

4.1 *WDR62*

Traditionally, microcephaly was defined as a neurodevelopmental disorder characterized by markedly reduced size of the brain and cerebral cortex and profound mental retardation, but mostly with normal brain structure. However, simplified gyration, which was most usually associated with classical lissencephaly (the pachygyria-agyria spectrum), was also observed in patients with microcephaly and became known as microcephaly with simplified gyration (MSG, [46-48] or in very severe cases, microlissencephaly [49, 50]. Lissencephaly is characterized by abnormal organization of the cortical layers but normal brain volume and is therefore regarded as a disorder of neuronal migration. More recently, there has been increasing awareness that patients with defined MCPH also have reduced cortical gyration resembling pachygyria . The first surprise and one of the first studies employing NGS to this field, came with the publication of mutations in *WDR62*, mapping to the MCPH2 locus on chromosome 19q13 in patients with microcephaly and a wide range of cortical malformations, including pachygyria, polymicrogyria, schizencephaly and cerebellar hypoplasia [17]. Knowledge of linkage of the MCPH2 locus to chromosome 19q was exploited by three groups who all used targeted NGS in custom arrays developed for the linked chromosome 19q13 region, in a few patients from families showing linkage to the region, to identify mutations in the *WDR62* gene. Using this approach in single patients from four families with MCPH linked to the region, Nicholas et al.

[16] identified different homozygous mutations in *WDR62*, including four missense mutations, an unusual finding in MCPH-associated genes. Yu et al. [18] used a similar approach in two affected individuals, using a custom array designed to span a 148 kb linkage interval, while Bilgüvar et al. [17] performed whole genome genotyping in two affected family members, followed by WES of targeted homozygous regions in a single patient. The analysis in further patients revealed six more homozygous mutations in WDR62 providing convincing evidence as the gene mutated in MCPH2. WDR62 encodes a 1,523 amino acid protein with multiple WD40 repeats, with little initially known about its function. Immunofluorescent staining of endogenous WDR62 in HeLa cells revealed a cell cycle-dependent localization, with spindle pole localization at the M phase. Its subcellular localization closely matched that of the centrosomal protein CEP170, which supported a predicted co-localization via a proteomic study. WDR62 was found to be localized to the apical margin of neuroepithelium cells and it showed a subcellular co-localization with ASPM, at the spindle poles during mitosis. These studies strongly suggested a function for WDR62 in cell proliferation and spindle formation, as well as in neuronal migration, thereby suggesting a common mechanism underlying primary microcephaly and other cortical malformations. Post-mortem analysis of a 27-week old foetus with mutations in *WDR62*, revealed a cortical pattern not dissimilar from that seen in *LIS1*-lissencephaly and *DCX*-subcortical heterotopia [18]. Mutations in *WDR62* are now known to be second most common cause of MCPH after mutations in *ASPM* (MCPH5).

4.2 *NDE1*

In a further study illustrating the overlap between cellular processes causing lissencephaly and severe microcephaly, Alkuraya et al. [51] and Bakircioglu et al. [52] demonstrated that truncating mutations in the C-terminal domain of *NDE1* were shown to cause a severe microlissencephaly syndrome resembling that described initially by Norman and Roberts [49, 50]. Whereas Alkuraya et al. used classical linkage analysis and Sanger sequencing of candidate genes to identify *NDE1* as the disease-causing gene in two consanguineous families, Bakircioglu et al. used a combination of

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autozygosity mapping (two families) and WES in a single patient from another family [52]. NDE1, originally identified as a critical regulator of a nuclear migration pathway, contains a homodimerisation domain as well as a highly conserved C-terminal NUDE_C domain, which allows its interaction with the dynein complex and is essential in centrosomal localization, mitosis and cell migration [51, 52]. The C terminus of NDE1 also includes a domain required for interaction with CENP-F, which directs NDE1 to kinetochores. Additionally, it was shown that Nde1 is phosphorylated by Cdk1 and that CDK1 phosphorylation at T246, within the C-terminal region that is disrupted by mutations in the patients they described, is required for cells to progress through the G2/M phase of mitosis[51].

Immunofluorescence via transfection of NDE1 constructs into HeLa mitotic cells demonstrated normal co-localization of NDE1 with γ-tubulin at the centrosome in wild-type constructs and abolished centrosomal and kinetochore localization in cells transfected with mutant constructs [52]. NDE1 expression was seen in the neuroepithelium throughout the developing human brain[52]. The effect of NDE1 deficiency on brain size appears to be much more striking in humans than in mouse and indicates defects in cortical lamination as well as neurogenesis [53]. Moreover, the cellular functions of NDE1 appeared to be either redundant or unnecessary in tested tissues, other than in the neuroepithelium, with localization of NDE1 to the mitotic spindle during metaphase being specific to neuroepithelial stem cells [52].

4.3 New genes, same phenotype

5.1 *CASC5* (MCPH4)

Jamieson *et al.* [54] initially reported linkage after homozygosity mapping in a Moroccon family with classic MCPH, to chromosome 15q14, and the locus received the assignment of MCPH4.

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Subsequently, the finding of mutations in the *CEP152* gene located within the linkage region defined by Jamieson et al. was reported [55]. It became clear that there are two genes mutated in MCPH within this region of chromosome 4 when Genin *et al*. reported the finding of mutations in the *CASC5* gene in their original Moroccan family and two further families[56]. These authors initially used Sanger sequencing and expression analysis on candidate genes within the linkage region in order to identify the disease-causing mutations in *CASC5*, however their subsequent analysis in order to eliminate the possibility of mutations in yet other genes within the region, involved targeted capture and NGS sequencing of the full genomic 2.7 Mb critical region on chromosome 15q. The CASC5 protein localizes to the kinetochore and performs two crucial functions during mitosis: correct attachment of the chromosome centromeres to the microtubule apparatus and spindle assembly checkpoint signalling. It interacts and binds to many other proteins essential to kinetochore function, including the proteins NDC80 and MIS12 referred to as the KMN (KNL1/Mis12 complex/Ndc80 complex) network [57]. It had previously been shown that *CASC5* knockdown in HeLa cells by siRNA caused a misalignment of chromosomes and premature entry into mitosis [58].

CASC5 binds to BUBR1 (BUB1B) via its N-terminal domain. BUB1B loss of function in humans causes Mosaic Variegated Aneuploidy (MVA) syndrome 1, an autosomal recessive microcephaly phenotype associated with a mitotic chromosomal segregation defect leading to aneuploidy for various chromosomes in fractions of cells in many tissues [59]. Therefore it is suggested that the primary microcephaly caused by defective CASC5 and BUBR1 results from a common mechanism involving chromosome missegregation. No aneuploidy, abnormal mitoses or other nuclear anomalies have been observed in lymphoblasts from patients with *CASC5* mutations [56], highlighting possible tissue-specificity for the expression of the defect.

5.2 *CDK6* (MCPH12)

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Homozygosity mapping after genome-wide linkage analysis in a consanguineous eight-generation family with seven affected and two unaffected individuals and parents identified a new MCPH locus on chromosome 7q21.11-q21.3 [27]. Despite having head circumferences ranging from -4 to -6 SD below the mean, patients had a relatively mild phenotype with mild intellectual disability. Sanger sequencing of the coding region of candidate genes within the linkage interval in four of the patients revealed a homozygous novel missense mutation in the *CDK6* (cyclin-dependent kinase 6) gene, which was also confirmed to co-segregate with the MCPH in the family. Since no further mutation in CDK6 was detected in additional MCPH families, the authors used post-hoc WES in a single patient in the family in order to demonstrate that no other mutations were present in other genes relative to the phenotype in the family.

CDK6 was previously known to demonstrate cytoplasmic and nuclear localization in various cell types [60]. Co-staining with the centrosomal marker, Pericentrin, showed that CDK6 surrounded pericentrin. Furthermore, CDK6 accumulated at the centrosome throughout the mitotic cycle, thereby resembling the distribution of WDR62 during mitosis. Analysis of patient fibroblasts revealed substantial abnormalities, including an aberrant microtubule network in interphase, abnormal spindle alignment during mitosis and misshapen nuclei. Some cells with misshapen nuclei had only a single centrosome, others had supernumerary centrosomes. Patient fibroblasts showed a reduced growth rate when compared to controls and fluorescence-activated cell sorting (FACS) analysis of patient and control primary fibroblasts detected a higher percentage of late-apoptotic patient cells [27].

A new role for centrosomal proteins in cell motility and polarity was observed via gap analysis in patient fibroblasts and cells in which CDK6 been knocked down [27]. Perturbation in the migration of neurons and glia cells has been suggested in patients with mutations in *WDR62* [18, 61] and *ASPM* [14]. Studies on the *LIS1* gene, which encodes a microtubule-stablilising protein acting primarily at

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the centrosome [62, 63], established the importance of centrosomal function in both neurogenesis and migration [64].

6 Same gene, new phenotype

6.1 *STIL* and Holoprosencephaly

Mutations in *STIL* were originally identified in patients with primary microcephaly (MCPH7, [65]. There have subsequently been only one report of mutations in *STIL* in patients with MCPH7 [66],bringing the total of MCPH-associated mutations to four. Thus *STIL* mutation is a rare cause of MCPH. STIL (SCL/TAL1 interrupting locus, also known as SIL) is a centrosomal protein that was identified as a candidate vertebrate homolog of the key centriole duplication factors SAS-5 in *C. elegans* and Ana2 in *Drosophila* [67]. It was subsequently shown to be an essential component of the centriole replication machinery in mammalian cells [68]. Overexpression of *STIL* results in excess centriole formation; whereas siRNA-mediated depletion of *STIL* leads to loss of centrioles and abrogates PLK4-induced centriole overduplication. Additionally, it was shown that STIL is necessary for SAS6 recruitment to centrioles, suggesting that it is essential for daughter centriole formation, interacts with the centromere protein CPAP and rapidly shuttles between the cytoplasm and centrioles [69]. Consistent with the requirement of centrioles for cilia formation, Stil–/– mouse embryonic fibroblasts lack primary cilia, a phenotype that was reverted by restoration of STIL expression [68]. Most recently, post-natal WES revealed two novel *STIL* mutations in a compound heterozygous constellation in a foetus with microcephaly and a structural brain anomaly consistent with holoprosencephaly [70]. Subsequently, WES from a single patient from an extended consanguineous family revealed another recessive *STIL* splice mutation in patients with microcephaly and lobar holoprosencephaly [71], thus confirming STIL mutation as a cause of holoprosencephaly.

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Holoprosencephaly (HPE) is a midline brain malformation that results from incomplete forebrain division. The HPE spectrum ranges from the severe, often lethal alobar type with no separation of the cerebral hemispheres, to semilobar HPE, the milder lobar HPE, the middle interhemispheric fusion variant, and finally to microform HPE with no overt brain malformations or neurologic findings [72]. Inactivation of *Stil* in mice causes embryonic lethality and midline neural tube defects, including holoprosencephaly-like malformations with a complete lack of midline separation at the anterior end of the cranial neural folds [73]. Taken together, these data suggest that *STIL* can be added to the list of HPE-causing genes in humans.

6.2 *CENPJ*, *CEP152* and Seckel syndrome

Both the *CENPJ* and *CEP152* genes were initially identified to cause primary microcephaly (MCPH6: [74] and MCPH4, later MCPH9[55], with patients showing an isolated brain phenotype. Mutations in both CEP152 and CENPJ have subsequently been identified in patients with Seckel syndrome [75, 76]. Independently, using a WES approach, the same Turkish founder mutation was identified in another patient with Seckel syndrome[75]. Both CENPJ and CEP152 are centrosomal proteins. CENPJ has been shown to have roles in centrosomal integrity, centriole duplication and elongation, mitotic spindle assembly/disassembly and mitosis [77-79]. It localises to the centrosome where it docks itself on the centrioles through PCNT, to facilitate mitotic spindle nucleation [80, 81]. Depletion of CENPJ resulted in arrest of cells in mitosis [77]. Thus, CENPJ apparently plays an essential role in the regulation of the cell cycle via its action on the centrosome. Additionally, CENPJ works as a transcriptional co-factor to STAT5 which plays an important role in regulating cell growth, differentiation, and survival [82].

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Analysis of the subcellular localization of *asl* (*asterless*), the *Drosophila* ortholog of *CEP152*, showed it to be associated with the periphery of centrioles, where it is involved in the initiation of centriole duplication [83]. Expression of CEP152 in HEK293T cells revealed fluorescence staining of the centrosomes, where it co-localized with pericentrin. The morphology of CEP152-deficient Seckel fibroblasts during interphase and during different stages of mitosis revealed that a substantial number of Seckel fibroblasts contained multiple, differently sized nuclei and centrosomes, micronuclei and fragmented centrosomes during interphase. During metaphase, CEP152-deficient Seckel lymphocytes showed aneuploidy in approximately 14% of metaphase spreads, with incorrectly aligned chromosomes, monopolar spindles with a single large centrosome, triple spindles with differently sized and structurally compromised centrosomes, and prematurely separated sister chromatids [75]. Most strikingly, CEP152-deficient cells appeared to be arrested at early anaphase in Seckel cells, compared to the wildtype population. The authors suggested that this block may have resulted from problems with chromatid alignment, uneven pulling forces in the spindle, or activation of a checkpoint that responds to weakly attached or misaligned chromosomes. Cell cycle analysis in CEP152 knockdown cells using short hairpin RNA suggested that CEP152 deficiency delays S-phase entry. Furthermore, fewer Seckel cells progressed to the G2/M phase and an increased proportion of cells stayed in G0/G1. These findings suggested altered ATR-mediated checkpoint activity and increased replicative stress in CEP152-deficient cells. CEP152 was found to be a binding partner of the protein CEP63, another centrosomal protein which had been previously implicated in mitotic entry and spindle formation [84, 85] and was subsequently implicated in MCPH [86]. CEP63 was found to be mutated in patients with MCPH and proportionate short stature, diagnosed as MCPH with growth retardation or mild Seckel syndrome [86].

Although mutations in the *CENPJ* gene appear to be very rare, the first three mutations identified (two frameshift truncating and a single missense mutation) all caused a phenotype of MCPH with normal growth (MCPH6 [74]). The mutation reported by Al-Dosari et al. [76] was a splice mutation

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which was shown at the mRNA level to result in the deletion of one to 3 exons. There is no simple explanation for the Seckel phenotype in these patients, but a plausible explanation provided by the authors was of possible brain-specific expression of transcripts affected by mutations that cause microcephaly without growth retardation.

6.3 *CENPE* (MCPH13) and Microcephalic Primordial Dwarfism (MPD)

Mirzaa et al. [87] reported a brother and sister, born of unrelated parents of European descent, with microcephaly, poor overall growth, and developmental delay. Both had intrauterine growth retardation and microcephaly apparent on prenatal ultrasound, as well as similar dysmorphic facial features, including sloping forehead, prominent nose, and mild micrognathia. At age 5 years, the older sib, a boy, had microcephaly (-9 SD), short stature (-7 SD), small hands and feet, mild spasticity, and severely delayed psychomotor development with absent speech and poor gross and fine motor skills. Brain imaging at age 17 days showed a diffuse, severely simplified gyral pattern with partial agenesis of the corpus callosum and cerebellar hypoplasia. Skeletal survey showed subtle widening of the ribs and possible metaphyseal areas of sclerosis in the distal femurs and proximal tibias. The hands appeared osteopenic, and the metacarpals were relatively short. Mirzaa et al. [87] noted that the skeletal findings were not characteristic of a specific bone dysplasia, particularly not of microcephalic osteodysplastic primordial dwarfism II (MOPDII), but suggested that those features may become more apparent with age. Additional features in the boy included well-controlled infantile seizures and congenital restrictive cardiomyopathy, which were not found in his affected sister. He died of pneumonia at age 8 years. At age 3 years, the sister had microcephaly (-7 SD) and mild short stature (-2 to -3 SD), as well as delayed psychomotor development, but not as severe as that in her brother. It was concluded that the phenotype was part of the spectrum that includes primary microcephaly and microcephalic primordial dwarfism [87].

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Using WES, followed by annotation of biological function and pathway membership of candidate genes harbouring two possibly pathogenic mutations, mutations in the *CENPE* gene were identified as the disease-causing mutations in the family [87]. CENP-E is required for spindle microtubule capture to the kinetochore, making these findings the first example of a core kinetochore defect contributing to MPD. In patient LCLs, mitotic cells exhibited a barely detectable CENP-E signal, as did PCNT-defective LCLs from a MOPDII patient. Impaired PCNT function mimicked impaired CENP-E function with respect to CENP-E localization, CENP-E-dependent BubR1 phosphorylation, mitotic spindle organization and mitotic progression. This indicated that spatially distinct proteins that play different roles in mitosis can manifest with similar cellular phenotypes.

6.4 Meier-Gorlin Syndrome and Microcephaly and Primordial Dwarfism (MPD)

Microcephalic primordial dwarfism (MPD), which is characterized by severely impaired growth beginning from early fetal life, is the defining feature of a group of disorders comprising several distinct disease entities, including Seckel syndrome [88], microcephalic osteodysplastic primordial dwarfism type II (MOPD II,[89] and Meier-Gorlin syndrome (MGS). Mutation of *PCNT* was reported in the context of two overlapping forms of primordial dwarfism, viz Seckel syndrome and MOPDII [90]. These disorders share common features of intrauterine growth retardation, severe postnatal short stature and marked microcephaly. Bone abnormalities are also common, however each disorder is distinguished by specific clinical features, e.g. MGS is characterized by severely reduced or absent patellae and small or abnormal ears. Recently, mutations in genes encoding ORC1, ORC4, ORC6, CDT1 and CDC6, which all encode proteins required for DNA replication origin licensing, were identified in patients displaying Seckel syndrome (SS) and/or Meier-Gorlin syndrome (MGS) [91, 92].

Eukaryotic chromosomal DNA replication involves coordinated initiation from multiple origins and has to be carefully regulated throughout the cell cycle. It is possible that much of this regulation

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occurs at the level of initiation of DNA synthesis. Replication in S phase of the cell cycle initiates from replication origins, which become "licensed" during the G1 phase [93-96]. Licencing begins with the binding of the origin recognition complex (ORC) followed by recruitment of the pre-replication complex (pre-RC) proteins, CDC6, CDT1 and the MCM2 to MCM7 helicase. The ORC comprises six components, ORC1 to ORC6, with ORC2 to 5 forming the core complex and ORC1 dissociating from the complex during the transition from the G1 to S phases [97]. There is increasing evidence that the ORC complex has additional important functions in heterochromatisation [98] and that ORC proteins localize to centrosomes [98], with siRNA depletion of ORC1 resulting in Cdk2 and cyclin E-dependent centriole and centrosome reduplication [99].

Cells from patients with Seckel syndrome can exhibit significantly impaired DNA damage response [100] [101]. While Seckel fibroblasts lacking ATR show no activation of H2AX upon UV- or hydroxyurea-(HU) treatment, which is essential in response to DNA damage and increased replicative stress, pericentrin-deficient cells derived from patients with MOPD II show no defects in ATR-dependent H2AX activation, but instead have defects in UV induced G2-M checkpoint arrest [100, 101]. Furthermore, Kalay et al. [75] recently reported that CEP152-deficient Seckel cells are more sensitive to DNA-damaging agents, and show an increased and prolonged activation of DNA damage response pathways[75].

Importantly Stiff T et al.[102] in investigations of cells from patients with MGS with regard to loss of origin licensing, found the surprising result that in addition to reduced licensing capacity in all cells and modest defects in centrosome and centriole copy number and organization, the greatest effect of siRNA on licensing components were marked defects in cilia formation. The findings of Stiff et al.[102] in cells in which *ORC1, ORC4, ORC6, CDT1*, and *CDC6* had been depleted resulted in dramatically impaired cilia formation, providing strong evidence that loss of origin licensing proteins substantially delays, although not fully ablates ability to form primary cilia. Thus impaired

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ciliogenesis was recognised as a novel pathogenic mechanism underlying the defects observed in patients with MGS.

7 New genes, new mechanisms

7.1 Microcephaly and cilia

Primary cilia are sensory organelles that nucleate from a basal body, made up of the mother centriole and associated pericentriolar proteins [103, 104]. They are found in most types of mammalian cells and function as mechano- and chemosensory organelles by using intraflagellar transport proteins to receive and transduce extracellular signals [105]. Cilia formation and centrosome/centriole biogenesis are overlapping and interdependent processes and therefore it can be expected that defects in centrosome function would affect cilia formation/biogenesis. The first hint that cilia dysfunction may be involved in microcephaly and growth retardation was the evidence that PCTN is associated with the basal body of primary cilia and is required for primary cilia biogenesis in olfactory cilia [106-108].

Mutations in centriole biogenesis have been reported in primary microcephaly and Seckel syndrome, disorders without the hallmark clinical features of ciliopathies. Very recently, mutations in the genes encoding PLK4 kinase, a master regulator of centriole duplication and its substrate and TUBGCP6, were identified in individuals with microcephalic primordial dwarfism and additional congenital anomalies previously associated with ciliopathies, including retinopathy, thereby extending the phenotypic spectrum associated with centriole dysfunction. PLK4 is a key regulator of centriole biogenesis [109, 110].

Individuals with *PLK4* mutations displayed profound microcephaly (OFC in order of -11.6 S.D) and substantial growth retardation of prenatal onset, with markedly reduced height in keeping with the diagnosis of microcephalic primordial dwarfism [111]. Neuroimaging revealed a marked reduction in

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cortical size with simplified gyration, brainstem and cerebellar hypoplasia. In addition, ocular anomalies were frequently observed, best summarised as severe generalized retinopathy. Whole exome sequencing of an unrelated patient with a similar phenotype including retinopathy revealed the patient to be homozygous for a frameshift mutation in *TUBGCP6* and screening of another 12 individuals with microcephaly and retinal dystrophy revealed *TUBGCP6* mutations in a further three patients. Interestingly, *TUBGCP6* is a direct phosphorylation target of PLK4 kinase, thus indicating that both proteins act in the same pathway to cause a microcephaly retinopathy phenotype. Homozygous *TUBGCP6* mutation had previously been reported in a single Amish patient with microcephaly and chorioretinopathy, after WES [112], which no doubt helped focus the search on *TUBGCP6*.

Based on the known role of PLK4 in centriole biogenesis, Martin et al. [111] investigated centriole biogenesis in patient-derived fibroblasts during mitosis. They found that mitotic spindle formation was affected in patient cells with reduced centriole number and monopolar spindles most frequently observed. The latter can be predicted to result in delayed mitotic progression and mitotic errors and increased cell death, although this was not observed in PLK4-mutant fibroblasts. Zebrafish depleted of plk4 transcripts via targeted splice-site blocking morpholinos showed significantly reduced overall body size, with the reduction in size correlating with the extent of transcript depletion. Further experiments to investigate the lower cell numbers in zebrafish embryos, indicated that increased apoptosis may also contribute to the lower cell numbers in these embryos. The authors postulated that a reduction in mitotic and cell cycle efficiency and the promotion of cell death may be sufficient to cause reduced organism size such as the observed dwarfism in patients.

The retinopathy phenotype was also investigated in plk4-morphant zebrafish, showing a loss of photoreceptors, as well as variable reduction in eye size, consistent with the ophthalmological findings in patients with *PLK4* and *TUBGCP6* mutations. A reduced number of ciliated cells was found in the photoreceptor layer in zebrafish and in serum-starved fibroblasts from affected individuals,

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with an absence of basal bodies correlating with the absence of cilia. Additional cilia-related phenotypes, such as left-right asymmetry, were observed upon the injection of higher doses of plk4 morpholinos in the zebrafish, accompanied by many cells having no remaining centrioles. This allowed the conclusion that the observed ciliopathy phenotype was most likely directly attributable to the absence of basal bodies.

Most recently, mutations in *CENPF* were identified in patients with mid-gestation fetal lethality, dysmorphic craniofacial features, cerebellar vermis hypoplasia, cleft palate, duodenal atresia and bilateral renal hypoplasia, in a non-consanguineous Caucasian family. MCPH was evident by mid-gestation in the affected foetuses. Candidate chromosomal regions possibly linked to the phenotype were identified by genome-wide SNP analysis in the family, followed by WES in a single affected and single unaffected family member. Two novel non-synonymous variants in the *CENPF* gene were identified to be the disease-causing mutations in the family, confirming the expected compound heterozygosity in the affected patients. Further WES in a large cohort of patients with primary microcephaly, identified two nonsense mutations (one common to the first family), in a patient with MCPH (OFC <-4 SD below the mean), associated with mild to moderate learning difficulties. The clinical findings in these patients suggested a ciliopathy disorder, which was reinforced by the finding of a basal body localisation of the CENP-F protein in ciliated fibroblasts. Moreover, CENP-F co-localised with Ninein at the sub-distal appendages of the mother centriole of mouse IMCD3 (kidney medulla/collecting duct epithelial) cells and zebrafish *cenpf* morphants displayed many features of a ciliopathy, including body axis curvature, laterality heart defects, hydrocephalus and pronephric cysts [113]. CENPF had previously been identified as a kinetochore protein [114] and to be associated with the Ndel1/Nde1/Lis1/dynein microtubule motor complexes [115]. It has also been shown to co-localise with IFT88 along the ciliary axoneme and co-migrate with other IFT-B components. CENP-F is dynamically expressed throughout the cell cycle, found at the kinetochore, stabilising the attachment of microtubules to the centromere, at early prophase until anaphase; and at the spindle mid-zone during early anaphases, before migration with cytoplasmic dynein-1 to the

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spindle poles. Depletion of CENP-F in vitro results in mitotic delay, failure of kinetochore assembly and misalignment of chromosomes in a subset of mitotic cells [116].

7.2 Microcephaly and chromatin-remodelling complexes

7.2.1 *ZNF335* (MCPH10)

The nuclear zinc finger protein, ZNF335/NIF-1, was identified as the causative gene for one of the most severe forms of MCPH so far described [117]. Patients with mutations in *ZNF335* had an OFC of nine SD below the mean and died within one year of age. Extreme microcephaly was accompanied by a severely simplified gyral pattern and subarachnoid fluid separating the brain and skull, thus indicating possible degeneration. Although the gene was identified as causing this severe microcephaly and neurodegeneration syndrome using conventional linkage and positional cloning, the mutation was confirmed and further characterized using mRNA-transcriptome sequencing (RNA-seq)[117].The trithorax (TrxG) and polycomb (PcG) chromatin-remodeling complexes work in opposition to activate or silence gene expression, respectively [118]. The TrxG complex regulates developmental expression of many genes that are important for patterning, cell proliferation, and stem cell identity, by maintaining genes in an active state [119]. The activation of gene expression by TrxG occurs via the methylation of lysine 4 on histone H3 (H3K4) [120]. Genetic ablation of ZNF335 led to early embryonic lethality in mice, and Emx1-Cre-driven knockout led to virtual absence of cortical structure[117]. Loss of ZNF335 in neurons caused premature cell-cycle exit of progenitors, leading to a precocious depletion of the progenitor pool. A critical downstream target of ZNF335 is REST/NRSF, representing a pathway that is critical for this neurogenetic function. These studies provided the first evidence for an upstream regulator of gene expression having an effect on the balance between progenitor cell division and differentiation.

7.2.2 *PHC1* (MCPH11)

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After linkage analysis and subsequent Sanger sequencing of candidate genes failed to identify the causative mutations and gene in a consanguineous family with two children affected with MCPH and four unaffected children, WES was performed on DNA from a single affected individual. This analysis successfully identified a single missense mutation in the *PHC1* gene which co-segregated with the MCPH in the family [121]. The affected individuals, who were homozygous for the mutation, had primary microcephaly (4.3 to 5.8 SD below the mean) with no additional structural brain abnormalities and mild intellectual disability. The PHC1 protein is part of a polycomb repressive complex (PRC1), the members of which function as transcriptional repressors that silence specific genes via chromatin remodelling. PRC1 members have been shown to play critical roles in the DNA repair pathway [122] and also interact with geminin, which has an established role in cell cycle control [123] [124] to promote its degradation. Awad et al. [121] showed reduced H2A ubiquitination, impaired recruitment of PHC1 to chromatin regions in response to DNA damage and reduced repair of DNA lesions in patient cells. This was the first report of a polycomb group gene (PcG) to be mutated in MCPH, and is reminiscent of the deficient DNA damage repair associated with mutation of the *PNKP* gene in patients with Microcephaly, Seizures and developmental delay (MCSZ, [125].

Conclusions

We have come a long way in a relatively short space of time from the original definition of primary microcephaly as a phenotype, to an improved understanding of the phenotypic spectrum of disorders which have primary microcephaly as the dominant clinical feature. The panoramic view of the genome provided by NGS technologies is not only providing us with novel insight into DNA variation, but also an open mind to phenotypes and their clinical classification. Although the concepts of locus heterogeneity and a single gene causing multiple syndromes are not new to

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human genetics, the extent to which these are being revealed through next-generation sequencing technology is remarkable.

Whole exome sequencing has rapidly expedited the identification of genes involved in MCPH, and the use of disease-specific gene panels for diagnostics is accelerating the time to genetic diagnosis for many patients and families. Just as array comparative genome hybridization (aCGH) exposed the considerable copy number variation in the human genome, the comprehensive nature of the new technologies has made us aware of the extensive sequence variability present in certain genes, even within the coding sequence. This is training us to refine our discriminatory powers for distinguishing variants based on their predicted pathogenicity. The accumulation of whole exome and whole genome data has stimulated efforts to create comprehensive public DNA variant and disease databases, which are constantly expanded and can be freely interrogated. Having started with the 1000 Genomes Project, we can now access not only variant databases with data from approximately 6500 exomes (National Heart, Lung and Blood Institute Grand Opportunity, NHLBI GO Exome Sequencing Project, ESP) and 60,706 exomes from the Exome Aggregation Consortium (ExAC Browser, http://exac.broadinstitute.org/), as well as databases combining variant and phenotype data with supporting evidence, e.g. ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/). We can now differentiate between genes that are tolerant of genetic functional variation and those which are intolerant and more likely to harbour mutations causing severe Mendelian disease, by calculation for example of their Residual Variance Intolerance Score (RVIS, [126]. Although this is an on-going process and we are by no means at the end of it, we have come a long way towards not only providing more genetic diagnosis but also more accurate diagnosis. At the moment most studies employing NGS technology are only looking at sequence variation and not as much effort has been directed towards complex structural variation (inversions, translocations, insertion/deletions, Alu repeats). The ability to reliably detect structural variation from NGS data is analytically challenging (Xie C and Tammi MT 2009) and will remain the task for the future. A further challenge remains in the development of more sophisticated methods of analysis for multigenic findings. A number of

these have already been reported, however their effect remains unknown , e.g.[127, 128] and it can be expected that there will be more to come. Further NGS technologies, such as RNA sequencing (RNA-seq,), chromatin immunoprecipitation in combination with NGS (ChIP-seq) and single cell genomics offer the potential to further explore the functional effects of mutations on the proteins and pathways in which they are involved, as well as address yet unanswered questions about the effects of MCPH proteins on brain growth and size.

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Table 1. Genes associated with primary microcephaly described in this article

Gene	**OMIM (gene)**	**Chromo-somal localization**	**MCPH design-ation**	**Phenotype**	**OMIM (pheno-type)**	**Technique used for gene identification**	**Original publication**
MCPH1	607117	8p23.1	MCPH1	Primary microcephaly	251200	Linkage analysis, positional cloning (linkage	Jackson *et al*. (2002)
WDR62	613583	19q	MCPH2	Primary microcephaly and MCD	604317	WES	Yu et al. (2010), Nicholas et al. (2010)
CDK5RAP2	608201	9q33.2	MCPH3	Primary microcephaly	604804	Linkage and subsequent positional cloning	Bond et al. (2005)
CASC5	609173	15q15.1	MCPH4	Primary microcephaly, mild to moderate ID	604321	WGL, homozygosity mapping, gene expression arrays, Sanger sequencing,	Genin et al. 2012
ASPM	605481	1q31.3	MCPH5	Primary microcephaly, mild to moderate ID	608716	Positional cloning	Bond et al. 2002
CENPJ	609279	13q12.12	MCPH6	MCPH and Seckel syndrome	608393	Linkage with subsequent positional cloning; WES	Bond et al. (2005), Al-Dosari et al. (2013),
STIL	181590	1p33	MCPH7	Primary microcephaly with mild to severe ID; microcephalic lobar holoprosencephaly	612703	Homozygosity mapping and positional cloning	Kumar et al. (2009), Borck et al. (2015)
CEP135	611423	4q12	MCPH8	Primary	614673	GWL followed by	Hussain et al.

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				microcephaly, severe ID, no speech		Sanger sequencing, post-hoc WES	(2012)
CEP152	613529	15q21.1	MCPH9	Primary microcephaly and Seckel syndrome	614852	Homozygosity mapping using SNP genotyping, Sanger sequencing	Guernsey et al. (2010), Kalay et al. (2010)
ZNF335	610827	20q13.12	MCPH10	Extreme microcephaly	615095	Linkage, positional cloning, RNA-seq	Yang et al. (2012)
PHC1	602978	12p13.31	MCPH11	Primary microcephaly, mild ID	615414	WES	Awad et al. (2013)
CDK6	603368	7q21.2	MCPH12	Microcephaly with mild ID	616080	WGL, homozygosity mapping, post-hoc WES	Hussain et al. (2013)
CENPE	117143	4q24	MCPH13	Microcephalic primordial dwarfism	616051	WES	Mirzaa et al. (2012)
CENPF	600236	1q41	-	Primary microcephaly with ciliopathy	NA	WGL and WES	Waters AM et al. (2015)
PLK4	605031	4q28.2	-	Microcephalic primordial dwarfism and chrorioretinopathy (MCCRP2)	616171	WGL linkage followed by WES in 1 affected	Martin et al. (2014)
TUBGCP6	610053	22q13.33	-	Microcephalic primordial dwarfism and chorioretinopathy (MCCRP1)	251270	WES	Puffenberger et al. (2012), Martin et al. (2014)
CEP63	614724	3q22.2	-	Microcephaly and short stature, Seckel syndrome 6	614728	Sanger sequencing of candidate genes	Sir et al. (2011)

ID: Intellectual disability; MCD: malformation of cortical development; NA: not available; WES: whole exome sequencing; WGL, whole genome linkage; RNA-seq: RNA sequencing

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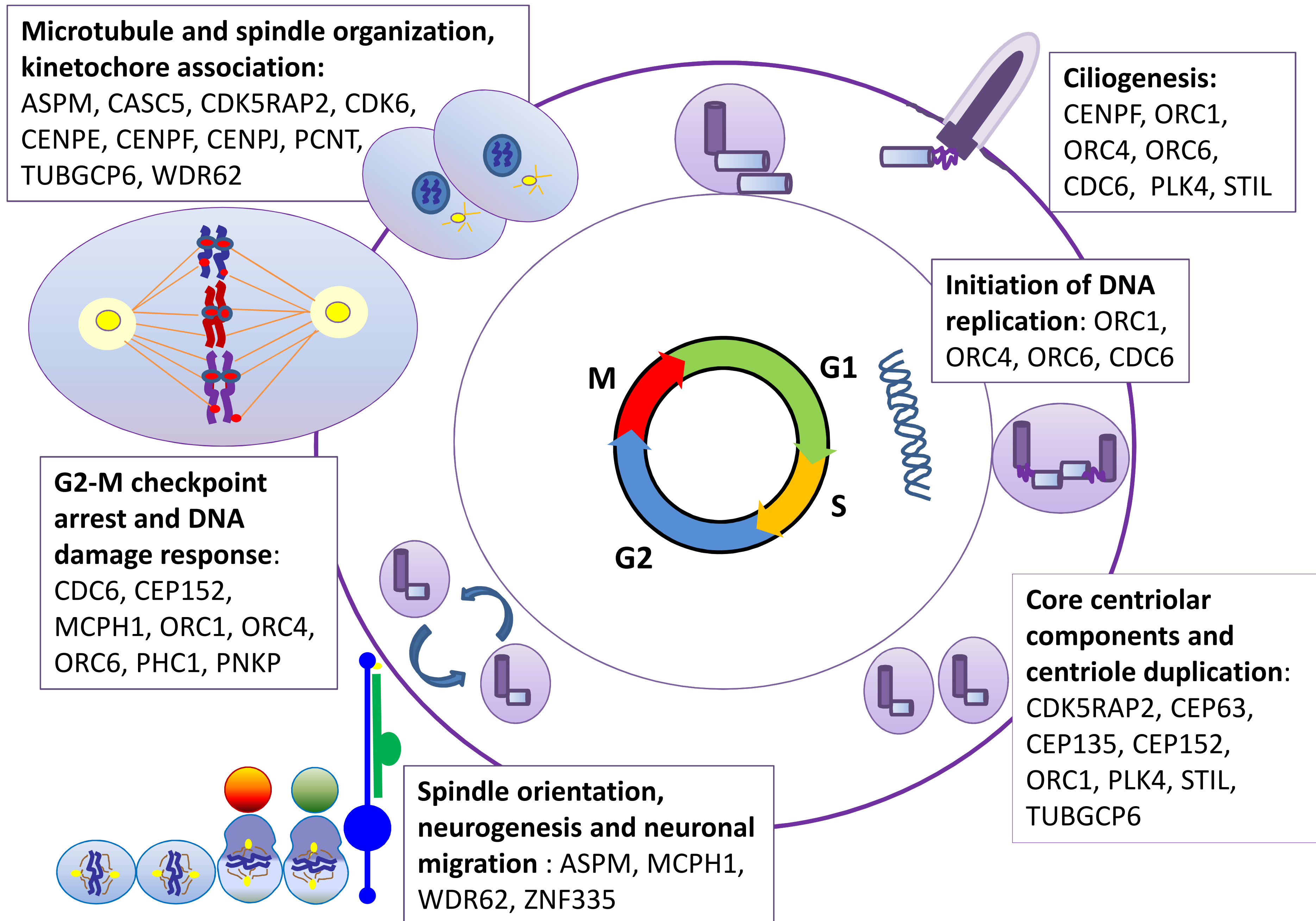

Microtubule and spindle organization, kinetochore association:
ASPM, CASC5, CDK5RAP2, CDK6, CENPE, CENPF, CENPJ, PCNT, TUBGCP6, WDR62
Ciliogenesis:
CENPF, ORC1, ORC4, ORC6, CDC6, PLK4, STIL
Initiation of DNA replication: ORC1, ORC4, ORC6, CDC6
M
G1
S
G2
G2-M checkpoint arrest and DNA damage response:
CDC6, CEP152, MCPH1, ORC1, ORC4, ORC6, PHC1, PNKP
Core centriolar components and centriole duplication:
CDK5RAP2, CEP63, CEP135, CEP152, ORC1, PLK4, STIL, TUBGCP6
Spindle orientation, neurogenesis and neuronal migration : ASPM, MCPH1, WDR62, ZNF335