Same but different: pleiotropy in centrosome-related microcephaly

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ABSTRACT **An intimate link between centrosome function and neurogenesis is revealed by the identification of many genes with centrosome-associated functions that are mutated in microcephaly disorders. Consistent with the major role of the centrosome in mitosis, mutations in these centrosome-related microcephaly (CRM) genes are thought to affect neurogenesis by depleting the pool of neural progenitor cells, primarily through apoptosis as a consequence of mitotic failure or premature differentiation as a consequence of cell cycle delay and randomization of spindle orientation. However, as suggested by the wide range of microcephaly phenotypes and the multifunctional nature of many CRM proteins, this picture of CRM gene function is incomplete. Here, we explore several examples of CRM genes pointing to additional functions that contribute to microcephaly, including regulation of cell cycle signaling, actin cytoskeleton, and Hippo pathway proteins, as well as functions in postmitotic neurons and glia. As these examples are likely just the tip of the iceberg, further exploration of the roles of microcephaly-related genes are certain to reveal additional unforeseen functions important for neurodevelopment.**

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INTRODUCTION

Centrosomes are supramolecular protein complexes critical for animal development, including formation and maturation of the most complex organ of all—the brain. Compelling evidence for a role in brain development stems from analysis of human patients that links mutations in at least 15 centrosome-related genes with a spectrum of microcephaly disorders (Table 1), including primary microcephaly (MCPH) and Seckel syndrome (SCKL), which have the common feature of reduced head and brain size reflecting fewer neurons (Duerinckx and Abramowicz, 2017; Nano and Basto, 2017). Centrosomes are multifunctional organelles, composed of pairs of centrioles surrounded by a dynamic pericentriolar matrix (PCM) of proteins, famous for their cell biological role as microtubule-organizing centers (MTOCs). In this capacity, the centrosome facilitates mitotic spindle formation, cell motility, intracellular trafficking, and immune synapse response, among other processes. Centrosomes also donate their core centriole structures to be repurposed as the basal bodies necessary for building motile and nonmotile cilia (Arquint *et al.*, 2014; Woodruff *et al.*, 2014; Lerit and Poulton, 2016; Vertii *et al.*, 2016).

Here lies the exciting mystery to be solved—linking the cell-biological roles of the centrosome with its roles in brain development. What precise neurogenic mechanisms are disrupted in centrosome-related microcephaly (CRM) mutants? Do different mutations in centrosome genes affect the same or different pathways? We highlight the complexity of the microcephaly disorder by showcasing commonalities and differences between phenotypes of centrosome MCPH and SCKL genes. Untangling CRM mutant contributions to the microcephaly phenotype requires expanding our current models.

FEWER NEURONS, SMALLER BRAIN

Microcephaly is defined by a reduction in brain size reflecting a reduction in the number of neurons. What then is the link between CRM mutations and loss of neurons? Is it simply that centrosomes

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Abbreviations used: CRM, centrosome-related microcephaly; MCPH, microcephaly primary hereditary; MTOC, microtubule-organizing center; NPC, neural progenitor cell; PCM, pericentriolar matrix; SAC, spindle assembly checkpoint; SCKL, Seckel syndrome.

Gene	OMIM	Functions	Common phenotypes	Variable phenotypes
WDR62	MCPH2	PCM, spindle integrity and orientation, Aurora A activation	Microcephaly, cortical malformations	Cortical malformations including pachygyria, cortical thickening, lissencephaly, subcortical band heterotopia, polymicrogyria, corpus callosum defects
CDK5RAP2	MCPH3	PCM, spindle orientation, centriole duplication, Hippo pathway regulation?	Microcephaly	Short stature, simplified gyral patterning, corpus callosum defects, hearing loss
ASPM	MCPH5	PCM, spindle integrity and orientation, regulation of actin cytoskeleton?	Microcephaly	Short stature, seizures, simplified gyral patterning
CPAP	MCPH6 SCKL4	PCM, centriole duplication, centriole growth, ciliary disassembly	Microcephaly, short stature (SCKL)	Seizures
STIL	MCPH7	Centriole duplication	Microcephaly	Holoprosencephaly
CEP135	MCPH8	PCM	Microcephaly	
CEP152	MCPH9 SCKL5	PCM, centriole duplication	Microcephaly, short stature (SCKL)	Simplified gyral patterning
CDK6	MCPH12	MTOC activity, cell cycle length	Microcephaly, simplified gyral patterning	
SAS6	MCPH14	Centriole duplication	Microcephaly	Seizures, abnormal ventricles, cerebellar hypoplasia
CEP63	SCKL6	PCM, centriole duplication, CDK1 recruitment	Microcephaly, short stature	
NIN	SCKL7	MTOC activity	Microcephaly, short stature	Immature sulcus patterning
TUBGCP4	MCCRP1	MTOC activity	Microcephaly, short stature	Eye defects, simplified gyral patterning
PLK4	MCCRP2	Centriole duplication	Microcephaly, short stature	Eye defects, simplified gyral patterning, small cerebellum and brainstem
TUBGCP6	MCCRP3	MTOC activity	Microcephaly, eye defects	Corpus callosum defects
PCNT	MOPD2	PCM, MTOC activity	Microcephaly, severe short stature	

TABLE 1: Centrosome-related microcephaly (CRM) genes.

are required for mitosis and thus disrupting centrosome function reduces the efficacy of cell division, resulting in fewer cells? To put this hypothesis in perspective, we briefly overview mammalian brain development.

The brain develops from a neuroepithelial tube of polarized neural progenitor cells (NPCs) with apical cilia extending into the ventricle (Figure 1; Dwyer *et al.*, 2016). NPCs progress through phases of cell division beginning with expansion of their numbers via symmetrical proliferative divisions. NPCs then begin to divide asymmetrically, generating one daughter that remains an NPC and one daughter that differentiates into an intermediate neural progenitor or a neuron that migrates basally. NPCs can also undergo a final symmetrical division to generate two neurons. The balance between proliferative and differentiative divisions is a key determinant of the final number of neurons in the brain. Current models of microcephaly mainly attribute the disorder to a reduction of the NPC pool, either through increased apoptosis or through premature differentiation. Therefore, understanding how centrosome function is linked to differentiation and apoptosis is key to understanding the roles of CRM genes in brain development.

The literature suggests a clear link. In NPCs, defects in spindle stability can cause prolonged mitosis and a delay in satisfying the spindle assembly checkpoint (SAC), leading to apoptosis (Chen *et al.*, 2014; Sgourdou *et al.*, 2017). Defects in cell fate and differentiation are also controlled, in part, by centrosomes through mitotic spindle misorientation (Li *et al.*, 2017), and through mother and daughter centriole inheritance (Wang *et al.*, 2009). Additionally, premature differentiation of NPCs can be triggered by improper centrosome-mediated cell cycle regulation (Capecchi and Pozner, 2015) or delayed ciliary disassembly (Gabriel *et al.*, 2016). In *Drosophila*, loss of both centrosomes and SAC causes increased cell death, premature differentiation, and a decreased proliferation rate of neural stem cells (Poulton *et al.*, 2017), pointing to the critical importance of mitotic functions in brain growth.

Thus, defects in centrosomes can increase both apoptosis and differentiation. This big-picture view is well substantiated, but many critical details remain unclear. It is also puzzling why the list of CRM mutations is not more expansive, including all genes critical for mitosis, differentiation, and apoptosis. As one investigates each CRM mutant in more detail, it becomes clear that the seemingly linear pathway to a smaller brain is much more complex.

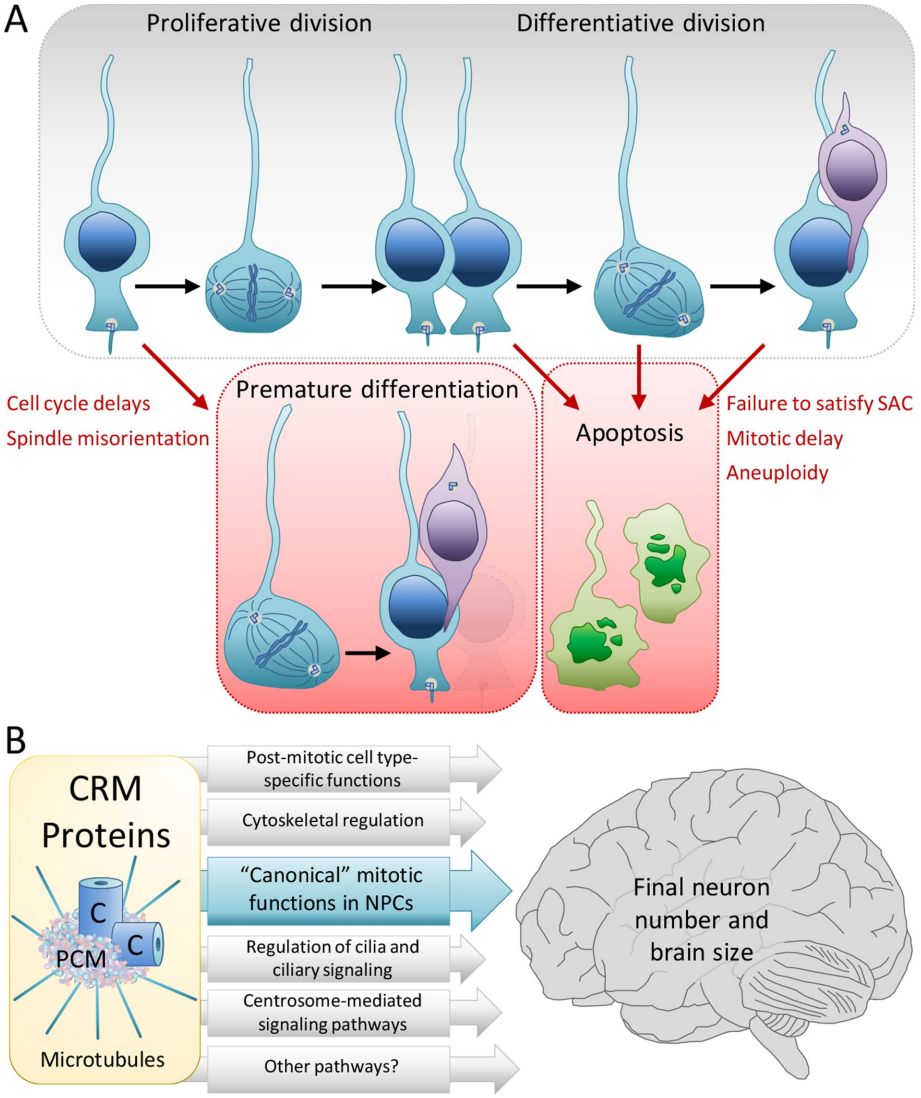

FIGURE 1: Canonical and noncanonical roles for centrosome-related microcephaly (CRM) genes in neurogenesis and brain size. (A) Neural progenitor cells (NPCs) undergo a series of symmetric proliferative divisions during early neurogenesis to expand the NPC pool. These cells then switch to an asymmetric mode of division that generates neurons and maintains the NPC pool throughout the later stages of neurogenesis (top). Defects in CRM genes can disrupt neurogenic divisions, resulting in loss of NPCs through premature differentiation due to spindle misorientation and cell cycle delays, or activation of apoptotic pathways due to failure to satisfy the SAC, mitotic delays, or aneuploidy (bottom). The end result of the depleted NPC pool is a reduction in final neuron number and ultimately brain size. (B) Schematic showing canonical mitotic functions for CRM genes (blue) and additional noncanonical roles (gray) that collectively contribute to proper neurogenesis and brain size.

CRM PROTEINS: BOUND TOGETHER, BUT FUNCTIONING INDEPENDENTLY

Centrosome proteins form a highly interconnected and dynamic network, allowing centrosomes to play many roles (Galletta *et al.*, 2016). This does not mean, however, that all centrosome proteins are required for all centrosome functions. In fact, many centrosome proteins have multiple cell type–specific and cell cycle–dependent roles, controlled by specific biochemical modifications and binding partners. For example, CPAP plays critical roles in centriole duplication (Tang *et al.*, 2011), spindle pole integrity (Chou *et al.*, 2016), and ciliary disassembly (Gabriel *et al.*, 2016). There are also several moonlighting roles for CRM proteins away from the centrosome. For example, *Drosophila Ana2* (*STIL*) functions both at the centriole (in procentriole formation) and away from the centriole at the cell cortex (in spindle pole orientation; Wang *et al.*, 2011). Therefore, while CRM proteins have clear overlapping functions, they are likely to participate in unique mechanisms or pathways that contribute to the control of brain size.

Independent roles for CRM genes are further supported by the observation that CRM mutations, in different genes or the same gene, cause MCPH with variant additional phenotypes. Some examples include *WDR62* mutations, which show several additional structural defects in the brain cortex (Bilgüvar *et al.*, 2010); *CPAP* mutations, which are associated with either MCPH or SCKL (Bond *et al.*, 2005; Al-Dosari *et al.*, 2010); and *CDK5RAP2* mutations, which are linked to MCPH, a more severe SCKL-like phenotype with deafness (Lancaster *et al.*, 2013), or a more minor defect affecting only the corpus callosum (Jouan *et al.*, 2016).

Collectively, these data suggest that many pathways are likely in play, and that a single model of neurogenic defects cannot explain all cases of CRM. To further probe this idea, we will next examine specific CRM genes to identify whether microcephaly is due to a role in differentiation, mitosis, apoptosis, or yet another unforeseen role.

ASPM: REGULATING THE ACTIN CYTOSKELETON TO CONTROL TISSUE ARCHITECTURE?

ASPM is the most commonly mutated CRM gene, accounting for 25–50% of all MCPH cases (Thornton and Woods, 2009). Mouse models of *ASPM* microcephaly have reduced cortical layers exhibiting premature differentiation of NPCs (Fish *et al.*, 2006; Capecchi and Pozner, 2015). Early studies in *ASPM*-depleted mice point to a defect in NPC spindle orientation with increased asymmetric divisions and a subsequent decrease in the progenitor pool as the primary mechanism underlying microcephaly (Fish *et al.*, 2006); subsequent work indicates that this model is incomplete.

More recently, ASPM was shown to regulate time spent in G1 by protecting Cyclin E from ubiquitin-mediated degradation, so that loss of *ASPM* can cause premature differentiation via cell cycle lengthening (Capecchi and Pozner, 2015). *Drosophila* mutants of the *ASPM* orthologue *asp* also have a smaller brain with spindle and cell division defects, suggesting a conserved function (Rujano *et al.*, 2013; Schoborg *et al.*, 2015). Interestingly, separation of function mutations show that reduced brain size is at least partially independent of spindle defects (Schoborg *et al.*, 2015). Instead, the reduced brain size in *asp* mutant flies is related to its role in regulating the actin cytoskeleton to control neuroepithelial architecture (Rujano *et al.*, 2013). These results are consistent with experiments in mice

showing that randomization of spindle orientation is associated with premature differentiation, but insufficient to cause reduction in cortical layers (Li *et al.*, 2017). Furthermore, *ASPM* mutant mice also exhibit disrupted apical epithelial architecture in the ventricular zone (Jayaraman *et al.*, 2016), suggesting that regulation of the actin cytoskeleton is a conserved mechanism contributing to proper brain size by Asp/ASPM. Thus, the prominent role of *ASPM* in spindle organization appears to play a relatively minor role in microcephaly. Exploring other roles for ASPM in more depth is a critical future research focus.

WDR62: A GLIAL-SPECIFIC FUNCTION IN MAMMALS?

WDR62, the second most commonly mutated gene in human MCPH patients, also appears to have unexpected additional roles beyond its function in NPC division, which might underlie microcephaly. WDR62 is best known for its functions in maintaining mitotic centrosome and spindle integrity by recruiting CPAP, both through a complex with CEP63 and ASPM, and through activation of Aurora A kinase (Chen *et al.*, 2014; Chou *et al.*, 2016; Jayaraman *et al.*, 2016). *WDR62* mutants have defective attachment of centrosomes to mitotic spindles, disorganized PCM, abnormal microtubule nucleation, and improper spindle orientation (Bogoyevitch *et al.*, 2012; Chen *et al.*, 2014; Ramdas Nair *et al.*, 2016; Sgourdou *et al.*, 2017). Furthermore, centrosome and spindle defects in *WDR62* mutant mouse NPCs prevent satisfaction of SAC and cause mitotic delay and apoptosis, leading to a reduction in cortical layers (Chen *et al.*, 2014; Sgourdou *et al.*, 2017).

In *Drosophila, Wdr62* mutants also have reduced PCM recruitment and show reduced brain size (Ramdas Nair *et al.*, 2016; Lim *et al.*, 2017), indicating conserved function. This work, however, shows a surprising deviation from the canonical *WDR62* function, as small brains in *Wdr62* mutant flies are linked to a deficit in postmitotic glial cells rather than neural stem cells. *Wdr62* depletion in neural stem cells is not sufficient to reduce brain size, whereas *Wdr62* depletion in glial cells causes loss of both glia and stem cells and reduced brain size, suggesting that glial signaling is necessary to maintain neural stem cell identity (Lim *et al.*, 2017). This glia-specific function depends on the interaction between Wdr62 and Aurora A, indicating further conservation between mammals and flies. Although glial cells have been shown to regulate mammalian NPC numbers (Cunningham *et al.*, 2013), it is unclear whether *WDR62* is involved in such processes; further studies are warranted.

CPAP: POSTER BOY FOR MULTIPLE PATHWAYS TO MICROCEPHALY?

CPAP is a multifunctional CRM gene, with roles in centriole duplication and elongation, PCM organization, and ciliary disassembly (Tang *et al.*, 2011; Zheng *et al.*, 2014; Gabriel *et al.*, 2016; Sharma *et al.*, 2016). Humans with *CPAP* mutations present with a range of phenotypic severity, and studies of various *CPAP* microcephaly models suggest distinct underlying mechanisms. For example, an MCPH CPAP variant with a single amino acid substitution in the TCP domain fails to localize efficiently to the centriole, fails to support centriole duplication, and is defective in recruiting several PCM components in cultured NPCs (Tang *et al.*, 2011; Zheng *et al.*, 2014). In contrast, NPCs in SCKL patient-derived organoids with a mutation deleting *CPAP*'s CC5 domain have proper centriole duplication, spindle morphology, and recruitment of key PCM components. However, their NPCs have defects in ciliary disassembly, and the increased time required to resorb the cilium causes a corresponding delay in the G1-S transition, leading to a loss of NPCs through premature differentiation (Gabriel *et al.*, 2016). *CPAP* null mutant mice have NPCs with normal spindle orientation, chromosome segregation, and interphase cell cycle progression; however, NPCs undergo increased apoptosis due to both prometaphase delay and premature differentiation (Bazzi and Anderson, 2014; Insolera *et al.*, 2014). Further, CPAP depletion in neurons impairs neuronal migration, revealing an additional postmitotic function for CPAP (Garcez *et al.*, 2015). Nonetheless, that such distinct mechanisms stemming from CPAP have all been implicated in microcephaly suggests that additional phenotypic complexity is masked by broad clinical definitions.

Given that ASPM, WDR62, and CPAP utilize novel cellular mechanisms in both mitotic and postmitotic cells to control brain size, a key question emerges—is it possible that other CRMs control brain size by mechanisms unrelated to their canonical cell division functions?

CDK5RAP2: A KEY REGULATOR OF THE HIPPO SIGNALING PATHWAY?

Disruption of PCM organizing and spindle pole focusing functions of CDK5RAP2 play a major role in *CDK5RAP2* mutant microcephaly (Fong *et al.*, 2008; Kodani *et al.*, 2015; Chavali *et al.*, 2016); however, recent work suggests the possibility of additional defects in centrosome-mediated signaling pathways, such as Hippo (Sukumaran *et al.*, 2017). Mutant *CDK5RAP2* patient–derived cells and *CDK5RAP2* mouse models link premature differentiation and apoptosis with a number of mitosis-related phenotypes, including defective centriole duplication, mitotic PCM disorganization, spindle misorientation, and aneuploidy (Buchman *et al.*, 2010; Lizarraga *et al.*, 2010; Lancaster *et al.*, 2013; Yigit *et al.*, 2015). Interestingly, however, CDK5RAP2 was recently shown to interact with Hippo pathway proteins, and *CDK5RAP2* MCPH patient-derived cells show altered Hippo pathway protein levels, indicating abnormal Hippo signaling (Sukumaran *et al.*, 2017). The Hippo pathway is a key regulator of cell proliferation, apoptosis, and organ size (Yu *et al.*, 2015). Further, many Hippo pathway components are apically localized (Yu and Guan, 2013), and in *Drosophila* neural stem cells, phosphorylation by the Hippo pathway kinase Warts is required for the localization of some apical complex proteins (Keder *et al.*, 2015). Disruption of Hippo signaling could potentially affect cell polarity and prevent proper localization of apical cell fate determinants, thereby altering cell fate decisions. Thus, Hippo signaling is well situated to play additional roles in determining brain size. Given the proposed roles of centrosomes and cilia as major centers of signal transduction (Arquint *et al.*, 2014), signaling pathways converging on the centrosome are likely to contribute to CRM in some mutants as well.

CONCLUDING REMARKS

Microcephaly is an extremely complex disorder, with nearly 30 genes linked to it to date. Many of these genes encode proteins, which can be classified into several broad functional groups, including DNA damage response, centromere organization, cell cycle control, chromatin regulation, and centrosome-related proteins. At first glance, CRM proteins seem to be the easiest class to investigate, given their role in mitotic spindle formation and its link to premature NPC differentiation and apoptosis. However, this model has fallen out of favor in light of studies showing that cell fate determination can be altered without causing microcephaly (Li *et al.*, 2017). The likely explanation is a complex blend of mitotic and nonmitotic function for CRM genes in both progenitors and postmitotic cells during brain development.

Characterization of microcephalic mutants and identification of novel neurogenic mechanisms underlying the phenotype require research models with complex neurodevelopment, and thus animal models and cultured brain organoids are well suited to the task. Considering the substantial similarities between *Drosophila* and mammalian neurogenesis (Homem and Knoblich, 2012) and the apparently well-conserved roles of microcephaly-associated genes between these species, we anticipate that studies in simple model organisms will reveal gene functions important for microcephaly, especially given the wide range of genetic manipulations allowing interrogation of mitotic and postmitotic roles. Similarly, we anticipate that as cerebral organoid culture becomes increasingly standardized, reproducible, and accessible, it will become an immensely powerful system for elucidating mechanisms of neurogenesis. Such model systems are particularly useful because they allow testing of different mutant isoforms with a common genetic background. While patient-derived cells are certainly informative, genetic background effects are expected to be significant, especially since many patients are consanguineous. Recapitulating human mutations allows characterization of mutation-specific defects in neurogenesis; however, to identify and tease apart specific mechanisms that contribute to microcephaly phenotypes, experiments using separation of function mutations are required. Thus, the study of microcephaly-associated genes is an exciting field of research that is well suited to a combination of basic cell and developmental biological analysis, which promises to reveal a more complete picture of how complex pathways cooperate to give rise to our most complex organ.

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