

Molecular Causes of Primary Microcephaly and Related Diseases: *a report from the UNIA Workshop*

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Abstract

The International University of Andalucía (UNIA) Current Trends in Biomedicine Workshop on Molecular Causes of Primary Microcephaly and Related Diseases took place in Baeza, Spain, November 18-20, 2019. This meeting brought together scientists from Europe, the United States and China to discuss recent advances in our molecular and genetic understanding of a group of rare neurodevelopmental diseases characterized by primary microcephaly, a condition in which head circumference is smaller than normal at birth. Microcephaly can be caused by inherited mutations that affect key cellular processes, or environmental exposure to radiation or other toxins. It can also result from viral infection, as exemplified by the recent Zika virus outbreak in South America. Here we summarize a number of the scientific advances presented and topics discussed at the meeting.

Primary Microcephaly and related diseases: the role of cell fate

The meeting was opened by Dr. Andrew Jackson (MRC HGU, Edinburgh, UK) with a quote from Dr. L.S. Penrose from 1963; “..few descriptive terms in medicine have more vagueness than the diagnosis of microcephaly”. Dr. Jackson followed with a review of human diseases associated with microcephaly, in which he recognized many important contributions from scientists and clinicians going back to the 1920s, including Penrose, Van den Bosch, Majewski and Woods. He then discussed his lab’s own recent advances in understanding the role of mutations in the DNA methyltransferase *DNMT3A* that had been previously linked to macrocephalic overgrowth syndrome (Tatton-Brown et al 2014; Tatton-Brown et al 2018). While *DNMT3A* mutations in that syndrome led to decreased protein levels and loss of function, his laboratory identified new gain-of-function *DNMT3A* mutations in human patients with microcephalic dwarfism (Heyn et al 2019). These mutations impaired the ability of the PWWP domain of *DNMT3A* to bind to methylated histone H3K36, leading to increased methylation of developmental regulatory loci in place of their usual Polycomb mediated H3K27 methylation. These data clearly demonstrate that the fine tuning of *DNMT3A* activity is key to determining brain size. Dr. Jackson proposed that this proper regulation of cell fate likely plays a role in a number of neurological disorders caused by epigenetic regulators.

The theme of cell fate determination was further explored in several excellent talks, including that of Dr. Francisco Tejedor (Instituto de Neurociencias,CSIC-UMH,

Alicante, Spain), who demonstrated the versatility of *Drosophila* for studying fate decisions in the brain through his work on Minibrain (*Mnb*) (Tejedor et al 1995). *Mnb* is an orthologue of the human *DYRK1A* gene that is mutated in microcephaly (Møller et al 2008) and overexpressed in Down's Syndrome. In previous work, they demonstrated that transient *Mnb/Dyrk1A* expression in flies and mice could induce the cell cycle regulator p27 to promote terminal neuronal differentiation (Hämmerle et al 2011; Soppa et al 2014; Shaikh et al 2016; Shaikh and Tejedor 2018). Loss of *Mnb* function caused a loss of neuronal cells and disorganization of the *Drosophila* larval brain. It was shown that *Mnb* controls key cell fate transitions of neural progenitors by integrating cell cycle, Notch signaling and proneural factors, so that the key balance between proliferation and differentiation is lost when *Mnb* is absent.

Dr. Nathalie Spassky (IBENS, Paris, France) and her graduate student Gonzalo Ortiz-Álvarez presented recently-published and unpublished work examining the role of Geminin family proteins, *GEMC1* and *MCIDAS* (also called Multicilin), in cell fate in the developing brain. Using several sophisticated lineage tracing strategies, they showed that radial glial cells are the source of both ependymal cells and adult neural stem cells (NSCs) (Ortiz-Álvarez et al 2019; Lalioti et al 2019). Manipulation of Geminin or *GEMC1*, using engineered mouse models or transient overexpression, demonstrated that *GEMC1* favored the ependymal cell fate, while Geminin favored the production of NSCs. This further demonstrates the antagonism between Geminin and *GEMC1* or *MCIDAS* (Balestrini et al 2010; Pefani et al 2011; Ma et al 2014), and suggests it may play an important role in the regulation of symmetric and asymmetric divisions in the developing brain. A remaining question is why both *GEMC1* and *MCIDAS*, which interact with Geminin and activate E2F mediated transcription, are both required for the generation of multiciliated ependymal cells (Zhou et al 2015; Arbi et al 2016; Terré et al 2016; Lu et al 2019). Michael Lewis (IRB Barcelona, Spain), a graduate student from the Stracker lab, shared unpublished data examining their interactomes using BioID. This approach identified almost all of the factors known to be required for multiciliated cell differentiation programs, as well as potentially important differences in the binding of *GEMC1* and *MCIDAS* to chromatin remodeling complexes that may differentially regulate their transcriptional output.

Dr. Debra Silver (Duke University, Durham, North Carolina, USA) presented data on how defects in genes that encode parts of the exon junction complex component contribute to microcephaly (McMahon et al 2016). Focusing on the *Magoh* gene, she showed that *Magoh* deficiency led to problems in mitosis which altered the outcomes of radial glial progenitor divisions, with increased neuron production that was accompanied by elevated apoptosis (Pilaz et al 2016; Sheehan et al 2020). These phenotypes were modulated by p53 levels (Mao et al 2016). Dr. Roel Quintens (Belgian Nuclear Research Centre, Mol, Belgium) presented work on the effects of radiation exposure on brain development. Prenatal exposure to radiation causes microcephaly and severe mental disability in a dose-dependent manner (Verreet et al 2016). Following radiation exposure, Dr. Quintens reported cell death and gene expression changes reflective of premature neurogenesis, similar to those induced by disruption of *Magoh*. Loss of p53 prevented both cell death and premature neuronal differentiation. This indicates a p53-dependent mechanism by which alterations in the proliferation/differentiation balance in neuroprogenitors may contribute to microcephaly.

Replication stress in microcephaly and neurodevelopment

One established contributor to microcephaly is replication stress, a situation defined by stalled replication forks and the generation of single-stranded DNA that activates the DNA damage response. Dr. Grant Stewart (University of Birmingham, UK) reported new insights into the poorly understood SLF1 and SLF2 proteins that were shown to recruit the SMC5/6 complex, involved in homologous recombination mediated DNA repair (Räschle et al 2015). Dr. Agata Smogorzewska (Rockefeller University, New York, USA) shared her recent discovery that the RTF2 protein localizes to replication forks and its removal is required for the proper cellular response to replication stress (Kottemann et al 2018). Her new work links RTF2 to RNaseH2 and she proposed that RTF2 may be a candidate gene for Aicardi-Goutieres syndrome (AGS), an autoimmune disorder that has been previously linked to mutations in the *RNASEH2A*, *B* or *C* genes (Rice et al 2007). Dr. Travis Stracker (IRB Barcelona, Spain) presented data showing that the TLK1 and TLK2 proteins play important roles in suppressing replication stress and innate immunity (Lee et al 2018; Segura-Bayona et al 2019). *TLK2* mutations were recently linked to microcephaly in a distinct neurodevelopmental disorder characterized also by intellectual disability and autism spectrum disorder (Lelieveld et al 2016; Reijnders et al 2018; Töpf et al 2020). Dr. Stracker proposed that replication stress and innate immune

activation could play distinct roles in the pathology of these patients (Segura-Bayona and Stracker 2019). Together these talks highlighted the potential relevance of replication stress, and particularly replication fork processing, in the etiology of many microcephaly related disorders (Kottemann et al 2018; Taylor et al 2019). Some discussion was also raised about the potential prevalence of autoimmune activation amongst these diseases.

Centrosomes and cilia in microcephaly and neurodevelopment

Nearly half of the genes associated with autosomal recessive primary microcephaly (MCPH) encode proteins that localise to centrosomes and/or mitotic spindle poles. Dr. Song-Hai Shi (Tsinghua University, Beijing, China) shared unpublished data from his lab in collaboration with Dr. Kathryn V. Anderson, Dr. Alexandra L. Joyner and Dr. Bryan Meng-fu Tsou (Sloan Kettering Institute, New York, USA) demonstrating that the conditional deletion of the CEP83 protein, which localizes to centriole distal appendages (Yang et al 2018), leads to the inability of basal bodies to attach to the apical membrane of neural progenitor cells, causing a defect in progenitor membrane properties and an enlarged cortex (Shao et al 2020). This model uncovered a key role for the docked centrosome and cilia in preventing brain overgrowth.

Drs. Shi, Jackson, Anderson, Bazzi, Gergely, Stracker and others previously implicated the loss of centrosomes in microcephaly (Sir et al 2011; Bazzi and Anderson 2014; Insolera et al 2014; Marjanović et al 2015). Microcephaly caused by centrosomal gene mutations in mouse models is dependent on p53, although canonical p53 activation pathways involving the DNA damage response or p38 do not appear to be involved. Recent work from Dr. Andrew Holland (Johns Hopkins University, Baltimore, USA), and several other labs, identified the mitotic surveillance pathway using CRISPR/Cas9 screening approaches on cells treated with PLK4 inhibitors to provoke centriole loss (Fong et al 2016; Lambrus et al 2016; Meitinger et al 2016; Lambrus and Holland 2017). In response to mitotic delay, this pathway triggers p53 stabilisation and activation, and requires 53BP1 and USP28, both previously linked to the DNA damage response. Dr. Holland shared unpublished data demonstrating a clear role for this newly discovered pathway in promoting microcephaly in several mouse models with centrosome loss, but not in a model caused by DNA repair deficiency. These exciting new insights place the mitotic surveillance pathway at the centre of centrosome dysfunction-driven p53 activation that underlies the loss of neural progenitors in numerous primary

microcephalies and some Seckel Syndrome variants (Nigg and Holland 2018). The apparent difference between the roles of 53BP1 and USP28 in DNA repair- and centrosome dysfunction-linked microcephaly prompted interesting discussions about the possibility that different p53 activation pathways act across different human microcephalies, a point that has not yet been systematically examined.

Another protein implicated in mitotic timing and the response to centrosome loss, TRIM37, was discussed by Dr. Fernando Balestra (CABIMER, Seville, Spain) (Fong et al 2016; Lambrus et al 2016; Meitinger et al 2016; Lambrus and Holland 2017). *TRIM37* is mutated in Mulibrey Nanism, a disorder characterized by progressive growth delays and craniofacial abnormalities, but not neurological symptoms (Avela et al 2000). Dr. Balestra showed that loss of TRIM37 causes the appearance of increased centriole numbers. Using expansion STED microscopy, they identified abnormal filamentous structures containing the centrosomal protein Centrobin that accumulated in the absence of TRIM37. Previous work by several groups, including one of the organizers, Dr. Ciaran Morrison, has demonstrated that Centrobin plays key roles in regulating cilia formation (Gottardo et al 2015; Ogungbenro et al 2018). Future work will be need to clarify the nature of these structures and determine the direct targets of TRIM37's E3 ubiquitin ligase activity.

While activation of the checkpoint kinase CHK1 is typically linked to replication stress, Dr. Ciaran Morrison (National University of Ireland Galway) described a potential new role for CHK1 in primary cilium formation. Using reverse genetics and pharmacological means, he showed that CHK1-deficient human tissue culture cells have a significant defect in ciliogenesis. CHK1 kinase is regulated by the tumour suppressor and microcephaly gene product, MCPH1, and Dr. Morrison showed that centrosome amplification seen in MCPH1-deficient chicken DT40 cells after DNA damage was dependent on CHK1. Dr. Juan Alberto Marchal (University of Jaén, Spain) also explored the interplay between MCPH1 and CHK1, showing that the inability of MCPH1-deficient lymphoblastoid cell lines to overcome, or adapt to, the cell cycle delay imposed by catalytic inhibition of topoisomerase II is CHK1 dependent (Arroyo et al 2019). How MCPH1 controls CHK1 responses to genotoxic stresses remains to be determined in detail.

Dr. Zhao-Qi Wang (Leibniz Institute on Aging, Jena, Germany) expanded on published work that described dysregulation of cell cycle control by knockout of *McpH1* in murine neuroprogenitors with the analysis of mice in which gene targeting had removed the first BRCT phospho-protein binding domain of McpH1 (Liu et al 2017). These mice have a phenotype identical to the *McpH1* nulls, with defective neuronal development leading to microcephaly, testicular atrophy and defective ovary development. They also showed premature chromosome condensation, with apparently normal DNA damage responses. With these data highlighting the importance of the first BRCT domain in mediating MCPH1 functions, Martina Kristofova from the Wang lab (Leibniz Institute on Aging, Jena, Germany) outlined a Bio-ID strategy for identifying MCPH1 interactors that depend on the first BRCT domain.

Dr. Carol-Anne Martin (MRC HGU, Edinburgh, UK) provided further evidence for the potential involvement of chromosome condensation mechanisms in microcephaly. (Martin et al 2016). She presented details of a patient with mosaicism of a mutation in the gene encoding the NCAPD3 condensin protein. Interestingly, homozygous mutant mice with the same mutation also show reduced size, although the links between chromosome condensation and microcephalic syndromes remain an open question.

Mutations in the gene encoding the centrosomal protein *CEP135* were previously implicated in microcephaly (Hussain et al 2012). Graduate student Jose González Martínez (Marcos Malumbres lab, CNIO, Madrid, Spain) presented the characterization of *Cep135* hypomorphic and loss-of-function alleles generated using CRISPR/Cas9 in mice. Similar to what has been observed in some other centrosome mutants, mice had reduced cortical size accompanied by elevated p53 levels and cell death. While the knockout of p53 suppressed apoptosis, surprisingly it did not rescue the brain size defects of the mice, indicating a more complex phenotype. Immunohistochemistry and electron microscopy revealed clear defects in centrosome duplication and monopolar spindles, as well as the appearance of phase dense cytoplasmic bodies. Ongoing work is aimed at better understanding the role of CEP135 in brain development.

Mitotic spindle defects, microcephaly and growth failure

While the duration of mitosis has clearly been implicated in microcephaly, Dr. Renata Basto (Institute Curie, Paris, France), shared new work demonstrating that unexpected

changes in microtubule density of the mitotic spindle during development also play a key role (Vargas-Hurtado et al 2019). Imaging of mitotic spindles at different stages of embryonic brain development revealed that they had thicker microtubule bundles at late stages (E16.5) of development. This change in morphology was at largely dependent on TPX2 and its ability to bind to both Eg5, a motor protein that moves on microtubules and Aurora kinase A, a key regulator of mitosis. This TPX2-dependent change in architecture enhanced spindle fidelity, providing an explanation for the enhanced sensitivity of E13.5 neural progenitors to mitotic challenges.

Dr. Sylvie Mazoyer (GENDEV, Lyon, France) discussed her work on the *RNU4ATAC* gene that is mutated in Taybi-Linder syndrome (TALS or MOPDI) (Putoux et al 2016; Cologne et al 2019). TALS patients, who have a short life expectancy, present with severe microcephaly, dwarfism and skeletal abnormalities, as well as immunodeficiency in less severe forms of the disease, such as Roifman Syndrome (Merico et al 2015). In contrast to the other protein coding genes discussed in the meeting, *RNU4ATAC* encodes a small nuclear RNA (snRNA) that is part of the U12-dependent minor spliceosome complex. Using zebrafish as a model system, the Mazoyer group showed that depletion of *rnu4atac* led to a complex set of severe phenotypes reminiscent of cilium dysfunction in zebrafish, as well as cardiac defects, microcephaly and brain hemorrhages. Using quantitative real-time PCR, they demonstrated a U12-specific intron splicing defect that impacted a number of known ciliary genes, potentially explaining the severe phenotypes.

The product of the large *ASPM* gene, which is mutated in a number of microcephaly patients, promotes the focusing of mitotic spindle poles (Létard et al 2018). Dr. Sandrine Passemard (Institute Curie, Paris, France) described how fibroblasts from 5 patients with mutations in different parts of *ASPM* were derived by iPS reprogramming and neural rosettes generated prior to organoid formation. These cells showed mitotic defects, providing additional evidence for the importance of appropriately-controlled spindle formation.

In addition to MCPH, centrosome dysfunction can cause primordial dwarfism, a type of proportional dwarfism. Dr. Fanni Gergely (Cancer Research UK, University of Cambridge, UK) discussed unpublished work from her group about the Cenpj mouse model of Seckel syndrome/primordial dwarfism (McIntyre et al 2012). Whereas live

imaging of early embryos reveals a small mitotic delay in most mutant cells, p53-dependent transcriptional activity and apoptosis are more prevalent in neural tissues, and accordingly, p53 deletion restored brain but not body size in Cenpj mutants. Hence, both the degree of p53 activation and its transcriptional outcome elicited by centrosome dysfunction are likely to be tissue specific.

Models of human brain development

A point raised by Andrew Jackson's opening remarks of the meeting was the significant difference between the size of human brains and that of the mouse, the most tractable vertebrate model for studying neurodevelopmental disorders. This substantial difference in brain size poses a challenge for modeling a number of human diseases and in several cases, neurodevelopmental disorders are not well recapitulated in mice. The advent of CRISPR/Cas9 approaches has made it possible to do targeted genetics in larger mammals, such as the ferret, and organoid technology has provided a new venue for studying human brain development (Lancaster et al 2013; Johnson et al 2018; Benito-Kwiecinski and Lancaster 2019). Human brain organoid technology has some drawbacks due to their heterogeneity and the complexity of the protocols. Dr. Jay Gopalakrishnan (University of Düsseldorf, Germany) shared new data on the large-scale production of more homogenous brain organoids in his laboratory, potentially solving some scaling issues that could improve the system for the screening of drug effectiveness on brain cancer, analysis of radiation damage and the development of new rare disease models using gene editing technology.

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