

RESEARCH ARTICLE

Digenic inheritance of human primary microcephaly delineates centrosomal and non-centrosomal pathways

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Abstract

Primary microcephaly (PM) is characterized by a small head since birth and is vastly heterogeneous both genetically and phenotypically. While most cases are monogenic, genetic interactions between *Aspm* and *Wdr62* have recently been described in a mouse model of PM. Here, we used two complementary, holistic in vivo approaches: high throughput DNA sequencing of multiple PM genes in human patients with PM, and genome-edited zebrafish modeling for the digenic inheritance of PM. Exomes of patients with PM showed a significant burden of variants in 75 PM genes, that persisted after removing monogenic causes of PM (e.g., biallelic pathogenic variants in *CEP152*). This observation was replicated in an independent cohort of patients with PM, where a PM gene panel showed in addition that the burden was carried by six centrosomal genes. Allelic frequencies were consistent with digenic inheritance. In zebrafish, non-centrosomal gene *casc5* $-/-$ produced a severe PM phenotype, that was not modified by centrosomal genes *aspm* or *wdr62* invalidation. A digenic, quadriallelic PM phenotype was produced by *aspm* and *wdr62*. Our observations provide strong evidence for digenic inheritance of human PM, involving centrosomal

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genes. Absence of genetic interaction between *casc5* and *aspm* or *wdr62* further delineates centrosomal and non-centrosomal pathways in PM.

KEYWORDS

complex inheritance, digenic inheritance, exome sequencing, primary microcephaly, zebrafish

1 | INTRODUCTION

Apparently Mendelian disorders can sometimes be better explained by an oligogenic inheritance model than by a canonical monogenic model, especially in the presence of incomplete penetrance, variable expressivity or locus heterogeneity (Gazzo et al., 2017). Digenic inheritance is the simplest form of oligogenic inheritance and refers to disorders resulting from pathogenic variants at two distinct loci (Lupski, 2012). True digenic inheritance requires the presence of variants at two independent loci to trigger the disease, while composite class inheritance refers to Mendelizing variants with modifiers (Papadimitriou et al., 2019). Examples of true digenic inheritance in human pathology include facioscapulohumeral muscular dystrophy Type 2 (Lemmers et al., 2012) or midline craniosynostosis (Timberlake et al., 2016). By contrast, dual molecular diagnosis refers to the aggregation, in a single individual, of two independent phenotypes, each caused by (a) variant(s) at a single locus (Posey et al., 2017). Dual diagnoses may be difficult to distinguish from digenic inheritance when the two phenotypes extensively overlap (Papadimitriou et al., 2019), but they are distinct from a functional point of view. Digenic inheritance, but not dual diagnoses, indicates a functional relationship between two loci, including protein–protein interaction, protein–DNA interaction, or a shared pathway, allowing for synergistic potentiation of alleles at the two loci (Schäffer, 2013). Digenic inheritance encompasses several models of allele combinations. The double heterozygosity model consists of heterozygous pathogenic variants at two loci. The triallelic model consists of biallelic pathogenic variants at one locus and a heterozygous pathogenic variant at another locus (Katsanis et al., 2001). The quadriallelic model consists of biallelic pathogenic variants at two loci. Evidence of digenic inheritance is provided by protein–protein or protein–DNA interaction, segregation of the phenotype in the family and/or functional studies including animal models (Gazzo et al., 2016). Genetic interactions have recently been described in a mouse model of primary microcephaly (PM) caused by *Aspm* and *Wdr62* (Jayaraman et al., 2016), possibly indicating a composite class of digenic inheritance.

PM is an important and heterogeneous group of disorders of brain development that results from insufficient production of mature neurons during neurogenesis. Human PM is characterized by a small occipito-frontal circumference (OFC) since birth, with a final head size in the adult ranging from -3 to -10 standard deviations (SDs) below the mean. Nonsyndromic (microcephaly primary hereditary [MCPH]) and syndromic (e.g., Seckel syndrome; Meier–Gorlin syndrome; MOPD2; PM with diabetes) forms are known. PM is mainly autosomal recessive, but the causing gene can be identified in fewer than 50% of patients. Some forms of PM are

furthermore associated with anomalies of neuron migration (Woods & Basto, 2014). PM is a model disease for the study of brain growth and of neuronal organization in the human cerebral cortex.

Eighteen genes have been reported to cause MCPH (Naveed et al., 2018). *ASPM* is the most common, followed by *WDR62* (Létard et al., 2018). Many PM-causing gene products, including *ASPM* and *WDR62*, are localized at the centrosome during interphase or spindle pole during mitosis and are hence believed to cause PM by a common, centrosomal mechanism (Barbelanne & Tsang, 2014; Megraw, Sharkey, & Nowakowski, 2011). The centrosome, however, fails to account for all PM-causing genes, leaving room for at least two other mechanisms (Duerinckx & Abramowicz, 2018). First, aberrant mitotic checkpoint activity, which could deplete the final number of neurons by reducing cell proliferation and initiating apoptosis (Zhou & Elledge, 2000), is the likely PM-causing mechanism with pathogenic variants in *CASC5*, directly required for the spindle assembly checkpoint (Kiyomitsu, Obuse, & Yanagida, 2007) or pathogenic variants in genes causing DNA replication stress (Zeman & Cimprich, 2014). Second, aberrant regulation of mRNA translation inducing apoptosis in neural progenitors is the likely mechanism of PM associated with a growing number of genes, including *TRMT10A* (Igoillo-Esteve et al., 2013).

In experimental PM, *Aspm* $-/-$ mice showed a modest reduction in brain size, which was strongly enhanced by an additional heterozygous *Wdr62* $+/-$ pathogenic variant. The quadriallelic *Aspm* $-/-$ *Wdr62* $-/-$ mice presented with embryonic lethality. Besides this genetic interaction, *Aspm* and *Wdr62* proteins were shown to physically interact at the mother centriole with the mediation of Cep63, highlighting the role of these proteins in centriole duplication, and presenting PM as a “centriolopathy” (Jayaraman et al., 2016).

It remains to be determined whether digenic/oligogenic inheritance of PM applies to humans or not, and it would be extremely interesting to demonstrate it. Better understanding the complex background of the PM phenotype would help to find a molecular diagnosis in a higher proportion of patients and would improve genetic counseling. Moreover, a systematic search for digenic or oligogenic inheritance has the potential to categorize mutually interacting genes, giving better insight on the cellular mechanisms implicated in PM, delineating several functional pathways, centriolar, and non-centriolar. In the present study, we aimed at better understanding the complex genetic background of apparently Mendelian PM. For this purpose, we used two complementary, holistic, in vivo approaches: high throughput DNA sequencing of multiple PM genes in human PM patients, and genome-edited zebrafish for modeling digenic inheritance of PM, crossing fish for binary combinations of three genes: two centrosomal (*aspm* and *wdr62*) and one non-centrosomal (*casc5*).

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

All procedures complied with the ethical guidelines of Hôpital Erasme –Université Libre de Bruxelles, whose Ethics Committee approved our study under reference P2016/199 (Ethics Committee Erasme Hospital, OMO21). Informed consent was obtained from the patient's representatives.

All zebrafish husbandry and experiments were performed under standard conditions in accordance with institutional (Université Libre de Bruxelles) and national ethical and animal welfare guidelines and regulation.

2.2 | Patients

The recruited patients with PM had an OFC smaller than 2 SD below the age- and sex-related mean at birth and/or smaller than 3 SD after age 1 year. They were referred to two reference genetic centers. Clinical information was obtained by the referring geneticist or pediatrician. The first patients' cohort (exome cohort) consisted of 47 PM patients and 140 control patients. The controls were in-house patients affected by non-neurological disorders: cardiac arrhythmia, renal graft tolerance, renal diseases, fertility problems, and also normal parents of normocephalic probands in a trio cohort. The replication cohort (gene panel cohort) consisted of 64 PM patients and 63 control patients, tested for recurrent fever syndromes. Indeed, our gene panel capture method included genes for PM, inherited fevers, as well as other pathologies including pulmonary arterial hypertension.

2.3 | High throughput sequencing

Patients' DNA samples from the exome cohort were enriched for exonic sequences, and patients' DNA samples from the gene panel cohort were enriched for exonic sequences of 14 PM genes as well as 10 other, non-neural genes. For exome sequencing, the DNA capture kit and the sequencing platform varied according to the reference genetic center and the time of the analysis. The different sequencing platforms were Beijing Genomics Institute, China (Illumina HiSeq 2000); AROS applied biotechnology, Denmark (Illumina HiSeq 2000); IntegraGen, Evry, France (Illumina HiSeq 2000); and BRIGHTcore BRussels Interuniversity Genomics High Throughput core, Brussels, Belgium (Illumina HiSeq 1500). The five DNA capture kits used were Illumina TruSeq ExomeTarget, NimbleGen Seqcap EZ v3, NimbleGen Seqcap EZ v5, Agilent SureSelect All Exon v1 and Agilent SureSelect All Exon v5. For the gene panel cohort, exonic sequences were enriched using SeqCap EZ Choice NimbleGen Roche, and sequencing was performed on a MiSeq Illumina sequencer at the molecular genetic laboratory of Erasme Hospital, Brussels, Belgium. The high throughput sequencing pipelines (exome and gene panel) are described in Supplemental Methods. The mean coverage per patient was 85X in the exome cohort, and >200X in the gene panel cohort.

2.4 | Variants pathogenicity classification

ACMG guidelines (Richards et al., 2015) were followed for variant pathogenicity classification. Pathogenic (Class 5) and likely pathogenic (Class 4) variants were considered as Mendelizing pathogenic variants. Familial segregation of the Mendelizing variants was checked using Sanger sequencing to demonstrate *trans* configuration in the autosomal recessive cases and de novo inheritance in the autosomal dominant cases. All variants reported in this manuscript have been submitted to the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>; ClinVar accessions SCV000998479-SCV000998508).

2.5 | Mutation burden tests

Variants were filtered for quality criteria (pass GATK (DePristo et al., 2011) standard filter, read depth ≥ 10), allelic frequency (based on the maximum minor allele frequency found in ExAC (Lek et al., 2016), 1,000 G (TheGenomes Project Consortium, 2015 1000), ESP6500 (<https://evs.gs.washington.edu/EVS/>; source: dbNSFP2.8), GoNL r5 (Genome of the Netherlands Consortium, 2014), ARIC5606 (<https://sites.csc.unc.edu/aric/>), and our in-house database), and for functional impact (nonsynonymous or splice junction effect, using snpeff_effect from SnpEff (Cingolani et al., 2012)).

Five different exome DNA capture kits were used for exome sequencing (see above). The intersection of the five different kits was examined, and only those variants included in the intersection of the bed files were considered for mutation burden testing. This approach reduced the total number of variants by a factor two.

PubMed (<https://www.ncbi.nlm.nih.gov/pmc/>) and Online Mendelian Inheritance in Man (<https://www.omim.org/>), as accessed on October 03, 2017, were used to select the genes known to cause human PM, establishing a set of 75 PM genes (ANKLE2, ARHGAP11B, ASPM, ATR, ATRIP, BLM, BRAT1, BUB1B, CASC5, CASK, CDC6, CDK5RAP2, CDK6, CDT1, CENPE, CENPF, CENPJ, CEP135, CEP152, CEP250, CEP63, CIT, COX7B, CTIP, DNA2, DONSON, DYRK1A, EFTUD2, EIF2AK3, ERCC3, ERCC4, ERCC5, ERCC6, ERCC8, IER3IP1, KIF11, KMT2B, LIG4, MCPH1, MFS2A2, MYCN, NBS1, NDE1, NIN, NIPBL, NSMCE2, ORC1, ORC4, ORC6, PCNT, PHC1, PLK4, PNKP, RAD50, RNU4ATAC, SASS6, SLC25A19, SLC9A6, SMC1A, SMC3, STAMBP, STIL, TRAI, TRMT10A, TUBA1A, TUBB, TUBB2B, TUBB3, TUBG1, TUBGCP4, TUBGCP6, UBE3A, WDR62, ZEB2, ZNF335). As controls, we used 1,926 housekeeping genes identified in at least seven different studies (detective breadth ≥ 7 ; Zhang, Akintola, Liu, & Sun, 2016). For the gene panel data, the selection was defined by our DNA capture kit targeting 14 PMs genes (ASPM, CASC5, CDK5RAP2, CENPJ, CEP135, CEP152, MCPH1, ORC1, ORC4, ORC6, PCNT, STIL, TRMT10A, WDR62), as well as non-neural genes involved in diagnostic work-ups of other pathologies, for example, pulmonary arterial hypertension. The 14 PM genes included six PMs genes expressed at the centrosome (ASPM, CDK5RAP2, CENPJ, CEP135, CEP152, and WDR62). Ten non-neural genes were selected as control genes (ACVRL1, BMPR1B, BMPR2, CAV1, ENG, KCNK3, LTBP2, SLC4A11, SMAD4, SMAD9).

Mutation burden tests were programmed in-house using R coding. For each patient, the number of allelic variants in PM genes was counted, homozygous variants counting as two allelic variants.

For each PM gene, the number of allelic variants in the case or control group was counted, and this number was divided by the number of patients in the group.

In a permutation test, 10,000 random subsets of 75 housekeeping genes were selected among the 1,926 housekeeping genes (Zhang et al., 2016).

2.6 | Zebrafish invalidations

Zebrafish orthology search and invalidation using transcription activator-like effector nucleases targeting *aspm*, *casc5*, and *wdr62* are described in Supplemental Methods. Zebrafish were maintained, bred, and raised at 28°C under standard conditions. For each targeted gene, at least two fish lines were obtained, harboring different pathogenic variants (Table S1).

2.7 | Zebrafish genotyping

The primers designed for the polymerase chain reaction are shown in Table S1. Detailed information on zebrafish genotyping is given in Supplemental Methods.

2.8 | Zebrafish live imaging

Live imaging was performed with a S8APO microscope (Leica), with a $\times 20$ magnification for the whole body pictures and a $\times 40$ magnification for the head pictures. Four to five days post fertilization (dpf) zebrafish larvae were anaesthetized in 0.02% tricaine, and placed on a Petri dish in a V-shaped 3% agarose mold (Adaptive Science Tools TU-1). Leica Application Suite V4.6 was used to analyze the pictures and take the measurements. Body length was the total length of the larva. Head area was measured on a dorsal view picture as previously shown (Brooks et al., 2014).

2.9 | Zebrafish experiments

Second generation (F2) heterozygous mutant fish were used for the experiments. First, each mutant line was studied separately to observe the phenotype of monogenic mutant fish. In a second step, F2 adult fish heterozygous for two different genes were crossed together and their progeny was studied. Part of the progeny was raised to adulthood to maintain the double heterozygous strains. In a third step, these double heterozygous fish were crossed together and their progeny was studied (Figure S1).

For each experiment, adult fish were crossed, four to five dpf zebrafish larvae were photographed and then directly put in tubes for genotyping. The association between phenotype and genotype was made *a posteriori*.

2.10 | Statistical methods

For the mutation burden test, the number of variants in the cases and in the controls were compared using a nonparametric Wilcoxon test with correction for ties. A permutation test with 10,000 selections of housekeeping genes was performed to exclude the effect of chance in

PM gene selection. A mutation burden was measured and the Wilcoxon statistic for independent samples was calculated for each of the 10,000 selections. The number of subsets of housekeeping genes yielding a Wilcoxon statistic higher than for the PM genes was counted, and this number divided by 10,000 was the *p* value of the permutation test.

For the zebrafish experiments, homozygous mutant larvae were compared to the heterozygous and to the wildtype larvae with the nonparametric Kruskal–Wallis test, followed by a Dunn's posttest.

The tests were considered statistically significant when $p < .05$. All statistical tests were two-tailed. They were performed using R software.

3 | RESULTS

3.1 | Patients with PM carry coding variants in 75 PM genes beyond Mendelian inheritance

We sequenced the exome of 47 PM probands. A monogenic cause of PM, for example, biallelic pathogenic variants in *CEP152*, was identified in the 75 PM genes in nine of the 47 patients (Table 1). We then compared the burden of variants in 75 PM genes, in the 47 PM patients and 140 control patients with non-neurological disorders. The variants were filtered as described in the Methods section, for various allelic frequencies (0.5, 1, 3, and 5%). A statistically significant burden was found in patients with PM, over the whole range of allelic frequencies (Table S2A). As this result did not rule out a strictly monogenic model of PM inheritance, we then removed from the analysis all the variants identified as monogenic causes of PM (16 alleles, see Table 1). Even without those causal variants, the patients with PM showed a higher number of allelic variants in the 75 PM genes than the control patients, for allelic frequencies of 5% or less (Figure 1), with a similar trend at smaller allelic frequency cut-offs. The statistics for each allelic frequency are shown in Table S2B.

In parallel, we measured the burden of allelic variants in 75 control genes (housekeeping genes) among PM and control patients (allelic frequency $< 5\%$), and observed no significant difference between the two groups (Figure 1). To exclude the effect of chance in the selection of the control genes, 10,000 permutations were performed with 75 randomly chosen housekeeping genes (allelic frequency $< 5\%$). Only 98 random selections of housekeeping genes gave a higher value of the Wilcoxon statistic than with the PM genes (*p* value of the permutation test 0.010). This means that we only have a 1% chance to be wrong in concluding that the difference between cases and controls was not due to chance in control gene selection.

Results from this first cohort thus showed that patients with PM carry a significant burden of variants in 75 PM genes, even after removal of the highly penetrant, Mendelizing variants.

3.2 | A replication cohort of patients with PM shows a burden of variants in six centrosomal genes

As a replication cohort, we then studied 64 unrelated patients with PM who underwent a diagnostic-grade panel sequencing of 14 PM genes with very high coverage (see Supplemental methods). Eight of

TABLE 1 Monogenic causes of PM; Mendelizing variants (Var), i.e., highly penetrant variants allowing for a Mendelian molecular diagnosis in patients with PM after exome analysis (75 genes inspected) or gene panel analysis (14 genes) are shown; biparental inheritance, or de novo inheritance, of every variant, was confirmed by Sanger sequencing in trios of probands and both parents

Patient #	Gene	Transcript; var	Zygoty	Protein	Inheritance	EXAC AF	gnomAD AF	CADD score	PhyloP100way_vertebrate	Reference
Exome #3	ERCC8	NM_000082.3; c.295_297delinsTG	Hom	p.R99Cfs*26	M/P	NR	NR	36,00	5,78	Rump et al, 2016
Exome #6	TRMT10A	NM_152292.4; c.379C>T	Hom	p.R127*	M/P	NR	NR	37,00	2,79	Igoillo-Esteve et al. (2013)
Exome #21	CEP152	NM_001194998.1; c.2878T>C	Het	p.W960R	P	2.5 × 10 ⁽⁻³⁾	2.6 × 10 ⁽⁻³⁾	19,23	6,70	NR
Exome #24	TUBA1A	NM_001194998.1; c.2959C>T	Het	p.R987*	M	8.5 × 10 ⁽⁻⁶⁾	1.2 × 10 ⁽⁻⁵⁾	42,00	2,24	Guernsey et al, 2010
Exome #28	CASC5	NM_006009.3; c.5G>A	Het	p.R2H	de novo	NR	NR	21,70	6,01	Gardner et al, 2018
Exome #32	KIF11	NM_170589.4; c.6123G>A	Hom	p.M2041I	M/P	8.3 × 10 ⁽⁻⁶⁾	4.02 × 10 ⁽⁻⁶⁾	15,09	1,56	Genin et al. (2012)
Exome #43	BLM	NM_004523.3; c.1116dup	Het	p.A373Sfs*4	de novo	NR	NR	NA	NA	NR
Exome #44	TUBGCP6	NM_000057.3; c.2650_2651del	Het	p.K884Afs*5	P	NR	NR	NA	NA	NR
Exome #45	LIG4	NM_000057.3; c.2809C>T	Het	p.Q937*	M	NR	NR	42,00	7,72	NR
Exome #45	LIG4	NM_020461.3; c.1075G>A	Het	p.V359I	P	NR	1.1 × 10 ⁽⁻⁵⁾	22,20	4,85	NR
Exome #45	LIG4	NM_020461.3; c.2968G>A	Het	p.G990R	M	NR	NR	2,89	-1,23	NR
Panel #1	ASPM	NM_002312.3; c.847 A>G	Het	p.K283E	M	NR	NR	14,87	5,91	Dard et al, 2017
Panel #12	ASPM	NM_002312.3; c.1271_1275del	Het	p.K424Rfs*20	P	1.5 × 10 ⁽⁻⁴⁾	1.6 × 10 ⁽⁻⁴⁾	NA	NA	Dard et al, 2017
Panel #14	WDR62	NM_018136.4; c.6513dup	Hom	p.V2172Sfs*7	M/P	NR	NR	NA	NA	Létard et al, 2017
Panel #14	WDR62	NM_018136.4; c.4195dup	Hom	p.T1399Nfs*20	M/P	NR	NR	NA	NA	Désir et al, 2008
Panel #15	ASPM	NM_001083961.1; c.1521G>A	Het	p.L507= (†)	M	2.8 × 10 ⁽⁻⁵⁾	1.3 × 10 ⁽⁻⁵⁾	NA	NA	NR
Panel #15	ASPM	NM_001083961.1; c.2788 C>T	Het	p.Q930*	P	NR	NR	19,16	2,00	NR
Panel #15	ASPM	NM_018136.4; c.1366G>T	Hom	p.E456*	M/P	NR	NR	26,90	1,76	Jamieson et al, 2000; Bond et al, 2003

(Continues)

TABLE 1 (Continued)

Patient #	Gene	Transcript; var	Zygoty	Protein	Inheritance	ExAC AF	gnomAD AF	CADD score	PhyloP100way_vertebrate	Reference
Panel #16	ASPM	NM_018136.4; c.4250_4251del	Het	p.Y1417*	P	NR	NR	NA	NA	Létard et al, 2017
Panel #17	ASPM	NM_018136.4; c.5590_5591del	Het	p.L1864Sfs*2	M	NR	NR	NA	NA	Létard et al, 2017
	ASPM	NM_018136.4; c.2389C>T	Het	p.R797*	P	8,3 × 10 ⁽⁻⁶⁾	1,2 × 10 ⁽⁻⁵⁾	43,00	4,21	Passemar et al, 2009
	ASPM	NM_018136.4; c.6686_6689del	Het	p.R2229Tfs*10	M	8,3 × 10 ⁽⁻⁶⁾	1,1 × 10 ⁽⁻⁵⁾	NA	NA	Passemar et al, 2009
Panel #59	CDK5RAP2	NM_018249.5; c.1376del	Hom	p.N459Ifs*7	M/P	NR	NR	NA	NA	NR
Panel #60	CASC5	NM_170589.4; c.6123G>A	Hom	p.M204I	M/P	8,3 × 10 ⁽⁻⁶⁾	4,02 × 10 ⁽⁻⁶⁾	15,09	1,56	Genin et al. (2012)

Note: *, splicing alteration (lymphoblasts).

Abbreviations: AF, allelic frequency; Het, heterozygous; Hom, homozygous; M, maternal; NA, not available; NR, not reported in literature; P, paternal.

the 64 patients were identified with a monogenic cause of PM, for example, biallelic pathogenic variants in *ASPM* (Table 1), and these variants were then removed from the analysis (15 alleles). Mutation burden tests compared the 64 PM patients to 63 control patients. The patients with PM had a higher number of allelic variants in six centrosomal PM genes for allelic frequencies of 1% or less (Figure 2). The statistics for each allelic frequency are shown in Table S3.

In parallel, a mutation burden test was performed with a subset of ten non-neural control genes and showed no significant difference between the cases and the controls (Figure 2).

Results from this second cohort confirmed the significant burden of PM gene variants in patients with PM and showed the burden to consist of variants in centrosomal genes.

3.3 | *Casc5* invalidation produces a severe MCPH/Seckel phenotype in zebrafish

To complement our approach in patients with PM, we aimed at modeling digenic inheritance in zebrafish, and at demonstrating digenic inheritance specifically with two centrosomal genes, as opposed to a centrosomal and a non-centrosomal gene. Therefore, we used genome editing to invalidate two centrosomal genes (*aspm* and *wdr62*) and one non-centrosomal gene (*casc5*), after showing that these genes were the sole orthologues of human *ASPM*, *WDR62*, and *CASC5* in the zebrafish genome (see Supplemental Methods). We first studied the phenotype in fish with homozygous premature termination codons in one of the three genes only. *Aspm* *-/-* larvae and *wdr62* *-/-* larvae showed a normal phenotype (*p* values after the Kruskal–Wallis test ranging from 0.536 to 0.795). *Casc5* *-/-* larvae showed a very severe phenotype, detectable from three dpf, and lethal after five to six dpf. The larvae had a small head and failed to thrive, with a short and incurvated body (Figure 3). After five to six dpf, edema developed and the *casc5* *-/-* larvae died. Five distinct experiments were performed on four to five dpf larvae, using two different *casc5* mutant lines (Table S1). In all five experiments, *casc5* *-/-* head area and body length were statistically significantly reduced in comparison to *casc5* *+/-* or wildtype larvae, as observed in the MCPH/Seckel type of human PM. One representative experiment is shown in Figure 3 (*p* values after the Kruskal–Wallis test, *p* < .001 for head area, *p* < .001 for body length). The *p* values after multiple comparison tests are shown in Table S4.

Our data showed that *casc5*-invalidated larvae had a severe developmental phenotype with microcephaly and short stature, while by contrast, invalidation of *aspm* or *wdr62* produced no apparent phenotype in zebrafish.

3.4 | *Aspm* and *wdr62* do not modify the *casc5* zebrafish phenotype

All the double heterozygous larvae had a normal phenotype (*aspm* *+/-* *wdr62* *+/-*, *aspm* *+/-* *casc5* *+/-*, and *casc5* *+/-* *wdr62* *+/-*), and no difference in the body length nor in the head area was

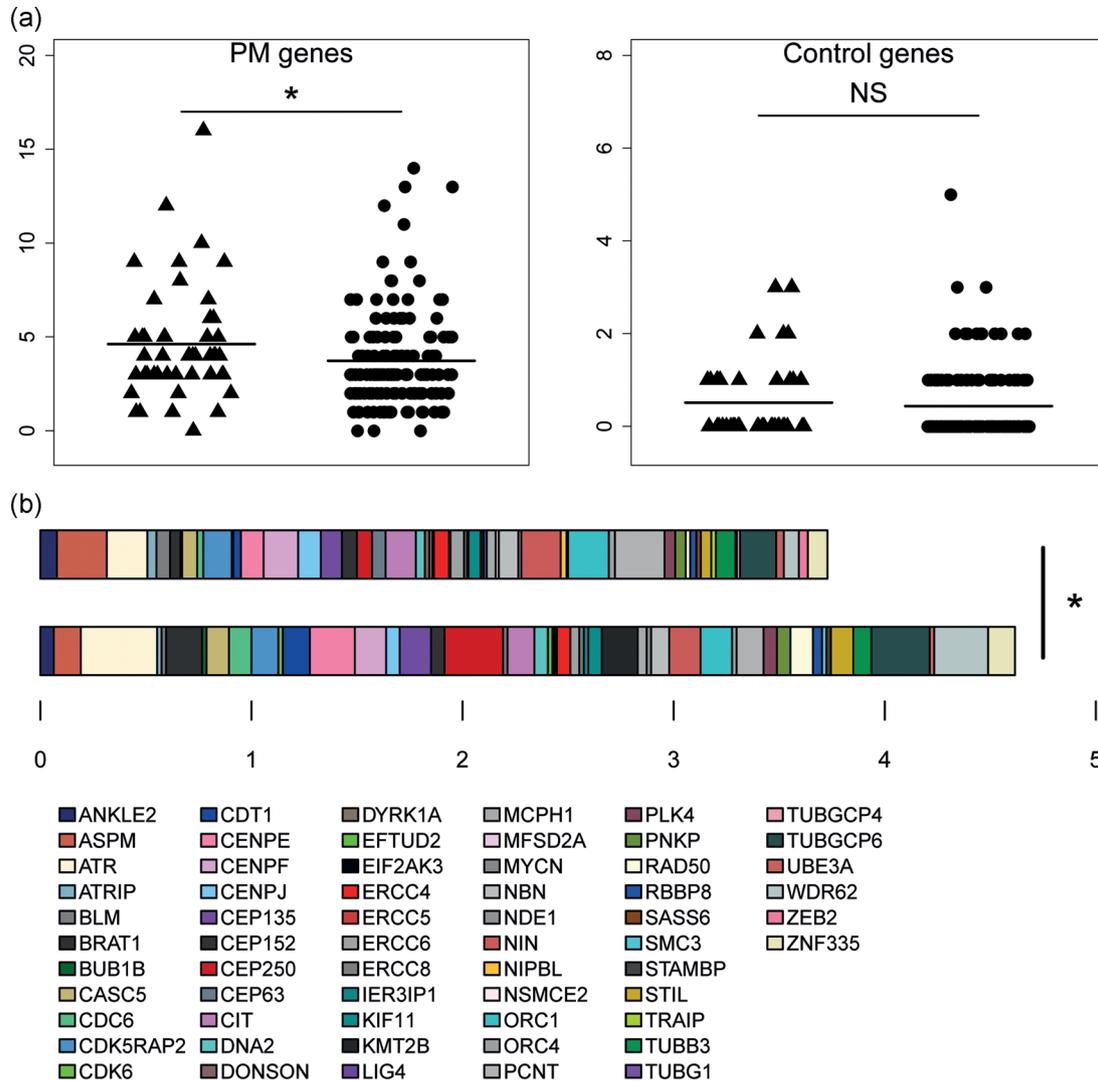


FIGURE 1 PM patients carry coding variants in 75 PM genes beyond Mendelian inheritance. (a) Variants in 75 PM genes (left) and in 75 control genes (right) identified via exome sequencing were filtered for allelic frequencies $<5\%$ in patients with PM (triangles, $n = 47$) and in control patients with non-neurological disorders (circles, $n = 140$). The y-axis shows the number of variants per patient. Variants identified as monogenic causes of PM in patients with PM (e.g., biallelic pathogenic variants in *CEP152*) were removed from the total count. Horizontal bars, mean number of variants. p Values after Wilcoxon test; $*p = .028$; NS, $p = .493$. (b) Mean number of allelic variants per patient in 75 PM genes, in the cases (bottom) and controls (top). The colors represent the distribution of the variants in the different genes. Genes containing no variant in either group were not represented. p Value after Wilcoxon test; $*p = .028$. NS, not significant; PM, primary microcephaly

observed in comparison with the wildtype larvae (p values after the Kruskal-Wallis test ranging from 0.317 to 0.970).

Aspm +/- *casc5* +/- crossings revealed an abnormal phenotype only in *aspm* +/- *casc5* -/-, *aspm* +/- *casc5* +/-, and *aspm* -/- *casc5* -/- larvae. The phenotype was the same as in the *casc5* monogenic experiments. All *casc5* -/- larvae had a short incurvated body, a small head, and died with edema after a few days. *Aspm* -/- *casc5* +/- triallelic larvae had a normal phenotype. One representative experiment out of three is shown in Figure S2 (p values after Kruskal-Wallis test, $p = .001$ for head area, $p < .001$ for body length). The p values after multiple comparison tests are shown in Table S5.

Casc5 +/- *wdr62* +/- crossings revealed an abnormal phenotype only in *casc5* -/- *wdr62* +/+, *casc5* -/- *wdr62* +/-, and *casc5* -/- *wdr62* -/- larvae. Here also, the phenotype was the same as in the *casc5* monogenic

experiments, all *casc5* -/- larvae had a short incurvated body, a small head, and died after a few days. *Casc5* +/- *wdr62* -/- triallelic larvae had a normal phenotype. One representative experiment out of three is shown in Figure S3 (p values after Kruskal-Wallis test, $p < .001$ for head area, $p < .001$ for body length). The p values after multiple comparison tests are shown in Table S6.

We thus observed that additional *aspm* or *wdr62* invalidation did not modify the *casc5* phenotype, whether heterozygous or homozygous.

3.5 | *Aspm* and *wdr62* produce a quadriallelic PM phenotype in zebrafish

Triallelic *aspm* +/- *wdr62* -/- and *aspm* -/- *wdr62* +/- larvae had a normal phenotype, while quadriallelic *aspm* -/- *wdr62* -/- had a very

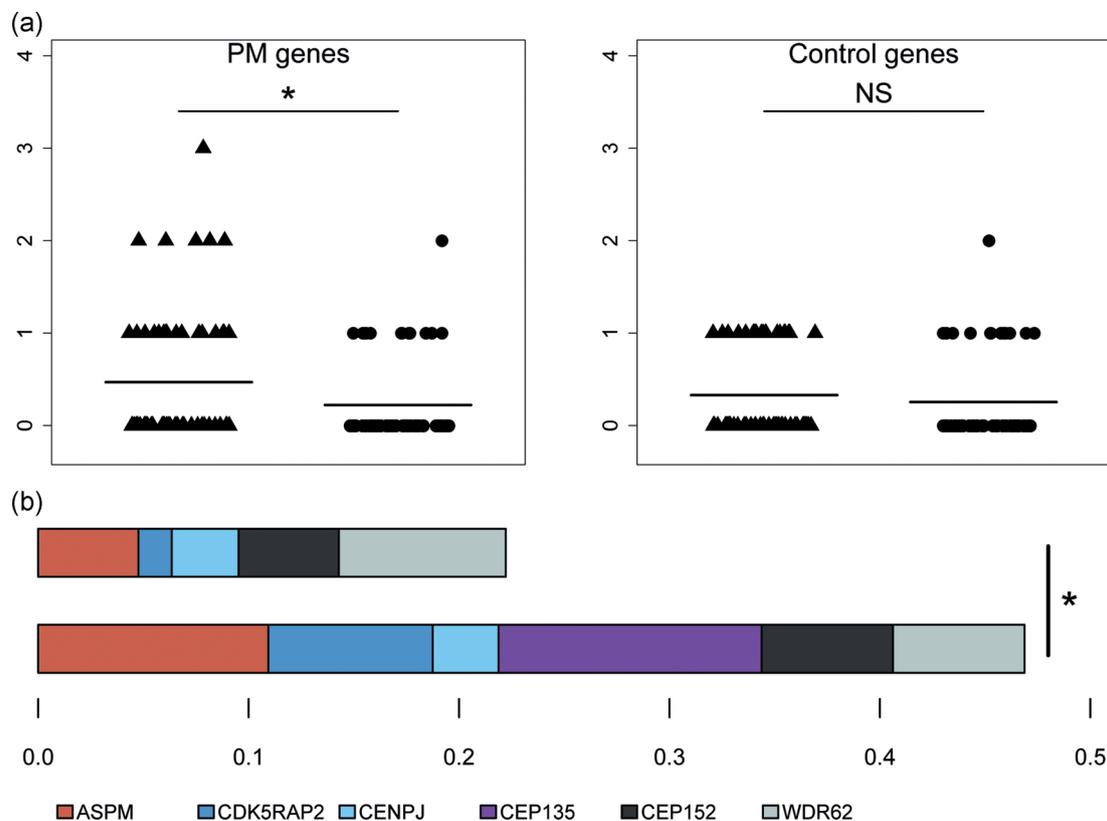


FIGURE 2 PM patients show a burden of variants in six centrosomal genes. (a) Variants in six centrosomal PM genes (left) and in ten control genes (right) identified via gene panel deep sequencing in patients with PM (triangles, $n = 64$) and control patients (circles, $n = 63$) and filtered for allelic frequencies $< 1\%$. The y-axis shows the number of variants per patient. Variants identified as monogenic causes of PM in patients with PM (e.g., biallelic pathogenic variants in *ASPM*) were removed from the count. Horizontal bars, mean number of variants. p Values after Wilcoxon test; $*p = .039$; NS, $p = .293$. (b) Mean number of allelic variants per patient in six centrosomal genes, in the cases (bottom) and controls (top). The colors represent the distribution of the variants in the different genes. p Value after Wilcoxon test; $*p = .039$. NS, not significant; PM, primary microcephaly

severe phenotype, lethal after a few days (Figure 4). Quadriallelic fish presented with a strong reduction in head size, and a shortened body length. One representative experiment out of three is shown in Figure 4 (p values after Kuskal-Wallis test, $p = .006$ for head area, $p = .018$ for body length). The p values after multiple comparison tests are shown in Table S7.

We thus observed a severe digenic phenotype in *aspm* $-/-$ and *wdr62* $-/-$ quadriallelic larvae, while both types of triallelic larvae displayed a normal phenotype.

3.6 | Candidate gene pairs from PM cohort digenic analysis

Finally, patients' results were re-examined individually, from a digenic viewpoint, in search of potential cases of double heterozygosity, triallelic, or quadriallelic inheritance. We observed candidate digenic pairs among centrosomal genes, including a case of triallelism with *ASPM* and *WDR62* variants, and cases of double heterozygosity and triallelism involving *CEP135* and *WDR62*, *CDK5RAP2* and *WDR62*, and *CDKRAP2* and *CEP135* variants. These candidate digenic pairs are listed in Table S8.

4 | DISCUSSION

PM is a usually Mendelian phenotype, often autosomal recessive, with vast genetic and phenotypic heterogeneity. We hypothesized that patients with identified or unidentified Mendelian pathogenic variants also carried a significant burden of less penetrant genetic variants in other PM-causing genes, that modified penetrance or expressivity.

We tested two independent cohorts of unrelated probands with PM in search for associations with genetic variants in PM genes beyond simple Mendelian inheritance, by measuring the burden of variants in a predefined set of PM genes, as compared with control patients with non-neurological disorders. A similar approach in patients with Charcot-Marie-Tooth has shown that a genetic burden contributed to phenotypic variability (Gonzaga-Jauregui et al., 2015). We found a significant burden of PM gene variants in patients with PM and then removed from the analysis all Mendelizing, highly penetrant variants identified as monogenic causes of PM (e.g., biallelic pathogenic variants in *CEP152*), to correct for the bias that patients with PM would obviously have more variants in PM genes because they harbor monogenic pathogenic variants in PM genes.

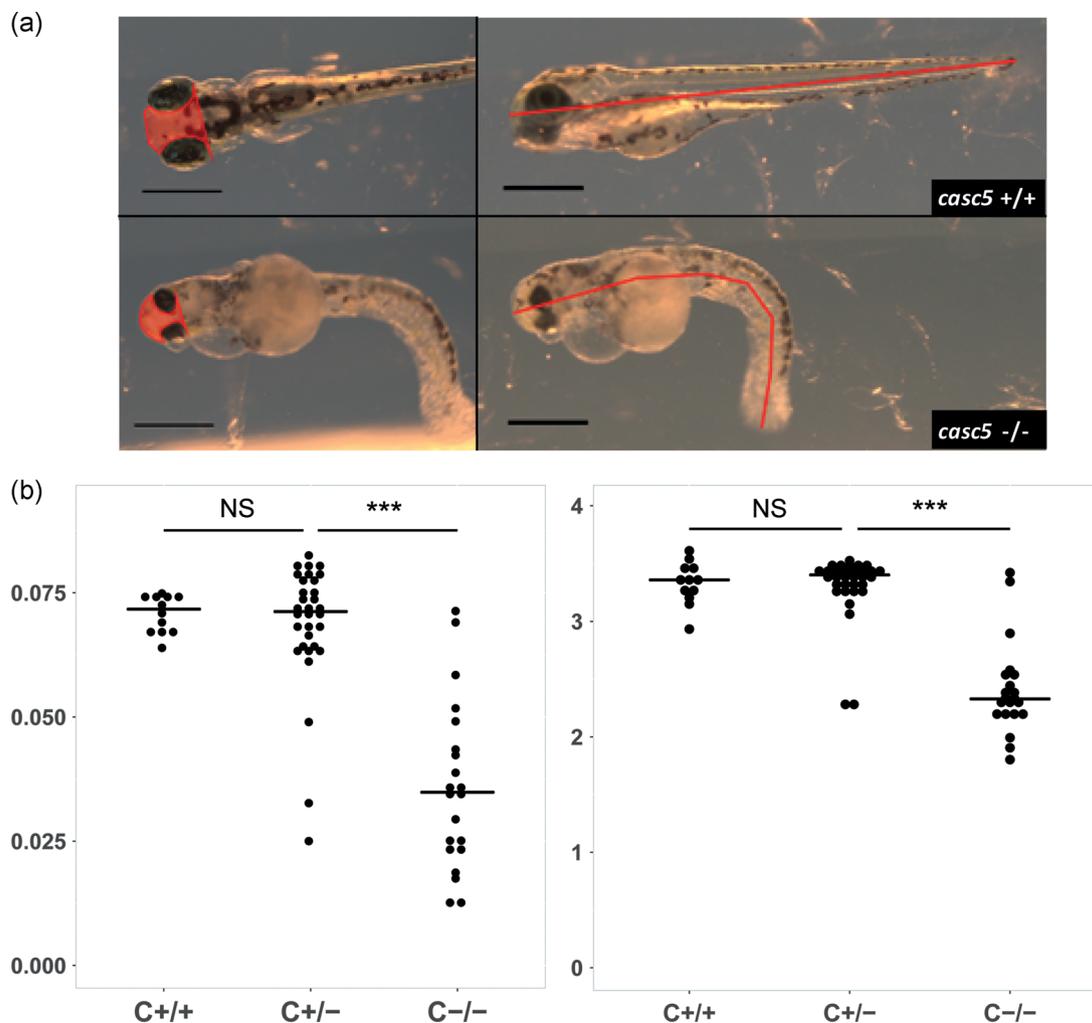


FIGURE 3 *Casc5* invalidation produces a severe MCPH/Seckel phenotype in zebrafish. (a) Live images of *casc5* +/+ (top) and *casc5* -/- (bottom) larvae. Dorsal views (left) show a reduced head size in *casc5* -/-. Lateral views (right) of whole larvae show a reduced body length in *casc5* -/-. *Casc5* -/- larvae are severely malformed and die after a few days. Bars = 500 μ m. (b) Head area (mm^2 , left) and body length (mm, right) of the larvae. $n = 76$. p Values after Dunn's posttest; *** $p < .001$; NS, $p \geq .050$. One representative experiment out of five. C, *casc5*; MCPH, microcephaly primary hereditary; NS, not significant

PM patients harboring only non-Mendelizing variants were also included in the analysis. In control experiments, we tested PM and non-PM patients for a burden of variants in non-PM genes, and observed no difference between the two groups.

In the exome cohort, patients with PM carried coding variants in 75 PM genes beyond Mendelian inheritance. Indeed, after monogenic causes of PM were removed from the analysis, an excess of PM gene variants persisted in the patients with PM in comparison with control patients, strongly suggesting a mode of inheritance more complex than simply Mendelian. Conversely, patients with PM and non-PM showed the same amount of variants in 75 randomly chosen control, housekeeping genes, and a permutation test confirmed that the significance we observed was not due to chance in the selection of the 75 control genes. The difference between cases and controls was highest when the PM variants were filtered for a 5% allelic frequency, which is compatible with oligogenic inheritance within the 75 PM genes (Manolio et al., 2009). In the gene panel cohort,

patients with PM showed a burden of variants in six centrosomal genes even after removing the variants identified to cause PM on a Mendelian basis. The difference between cases and controls was highest when the variants were filtered with a 1% allelic frequency, which is compatible with digenic inheritance within the six genes of the panel. Most variants involved in rare digenic diseases are rare (allelic frequency $< 1\%$) and their gene products interact directly or indirectly (Gazzo et al., 2016). Oligogenic or multigenic inheritance cannot involve rare variants only, because such combinations would be exceedingly rare (Manolio et al., 2009). Our observations thus suggest the existence of a digenic mode of inheritance in human PM, involving centrosomal genes.

Zebrafish studies were performed to validate digenic inheritance in another, holistic model, and to query molecular interactions between putative centrosomal or non-centrosomal PM genes. We chose *casc5* as a paradigm of the non-centrosomal gene. Contrary to *ASPM* and *WDR62*, *CASC5* is expressed at the kinetochore and is required for chromatin

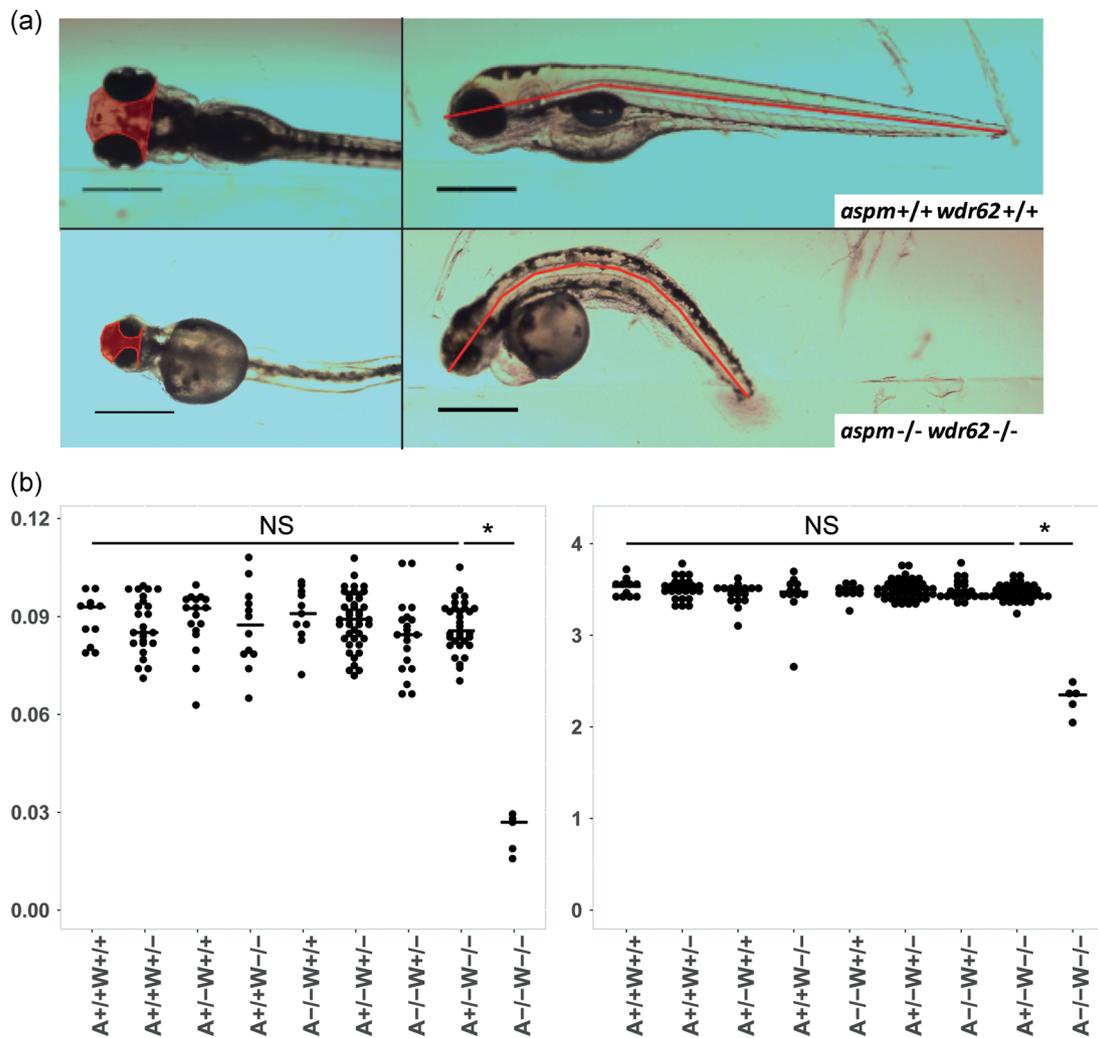


FIGURE 4 *Aspm* and *wdr62* produce a quadriallelic PM phenotype in zebrafish. (a) Live images of *aspm*^{+/+} *wdr62*^{+/+} (top) and *aspm*^{-/-} *wdr62*^{-/-} (bottom) larvae. Dorsal views (left) show a strongly reduced head size in *aspm*^{-/-} *wdr62*^{-/-}. Lateral views (right) of whole larvae show a reduced body length in *aspm*^{-/-} *wdr62*^{-/-}. Bars = 500 μ m. (b) Head area (mm², left) and body length (mm, right) of the larvae. $n = 177$. p Values after Dunn's posttest; * $p < .050$; NS, $p \geq .050$. One representative experiment out of three. A, *aspm*; NS, not significant; PM, primary microcephaly; W, *wdr62*

attachment to the mitotic apparatus (Kiyomitsu et al., 2007). First, we had to study monogenic inactivation of *casc5* in the zebrafish, which had not been reported. Our *casc5*^{-/-} zebrafish displayed a severe MCPH-like phenotype, consisting of a reduction in head size and body length, which was consistent with the human *CASC5* phenotype. Indeed, all patients with *CASC5* biallelic pathogenic variants presented with congenital microcephaly and short stature (Saadi et al., 2016). The phenotype in zebrafish was very severe, lethal after a few days, while the human patients survive into adulthood. This is compatible with the fact that the genome-edited zebrafish alleles were null, while the pathogenic splicing variants observed in patients likely result in hypomorphic alleles (Genin et al., 2012). To our knowledge, we here report the first zebrafish model for *CASC5* inactivation.

Our genome-edited *aspm*^{-/-} and *wdr62*^{-/-} zebrafish displayed a normal phenotype. However, knocking down each of these genes using morpholinos had previously produced a reduction in head and

eye sizes in zebrafish larvae (Kim et al., 2011; Novorol et al., 2013). This discrepancy may be due to a genetic compensation triggered by the gene inactivations but not by the morpholinos (El-Brolosy et al., 2019). Quadriallelic *aspm*^{-/-} *wdr62*^{-/-} were severely affected, demonstrating digenic inheritance, consistent with direct protein interaction at the centrosome as observed in mice (Jayaraman et al., 2016). Conversely, the *casc5*^{+/-} or *-/-* phenotypes were not modified by additional *aspm* or *wdr62* inactivation. Our observations thus provide human and zebrafish *in vivo* evidence for genetic interaction between *aspm* and *wdr62*, and absence of genetic interaction between either *aspm* or *wdr62* and *casc5*, consistent with distinct pathways being involved in the pathogenesis of PM.

The distinction between digenic inheritance and dual molecular diagnoses with extensively, if not completely, overlapping phenotypes (Posey et al., 2017) is not simple. We observed no patient with two definite molecular diagnoses in our cohort (e.g., Mendelizing,

biallelic pathogenic variants in *ASPM* and in *WDR62*), which indicates that dual diagnoses are unlikely to be frequent in patients with PM. Our findings hence support genuine digenic inheritance, either true digenic, or composite class digenic, inheritance (see below). Furthermore, the quadriallelic phenotype observed in *aspm* $-/-$ *wdr62* $-/-$ fish also indicates digenic inheritance, as both biallelic mutants had normal phenotypes. In humans, digenic inheritance might take the form of double heterozygosity within the cases that have no molecular diagnosis under the current Mendelian model and correspond to true digenic inheritance. Other patients harbor triallelic variants, consisting of the previously identified biallelic variants and an additional heterozygous variant at a second PM locus, suggesting composite class digenic inheritance. In the absence of the third variant, the biallelic variants would presumably produce a milder phenotype, and it would be interesting to test this hypothesis in mildly affected siblings of familial cases. Of note, some PM probands with double heterozygosity might in fact correspond to triallelism with a second, unidentified mutation at one of the two loci.

We replicated the patients with PM study in an independent cohort of patients with PM using a more focused genetic approach consisting of a PM gene panel analysis and confirmed an excess of variants in PM genes, which furthermore clustered within centrosomal genes. In this cohort, only 14 PM genes were sequenced, with a very high—diagnostic-grade—coverage ($>200X$ mean coverage), while in the exome cohort, the coverage was much lower (85X mean coverage) and varied across the exome.

We also performed a mutation burden test in the subset of six centrosomal genes in the exome cohort, but this was not significant, probably because of insufficient coverage resulting in an incomplete detection of variants in the exome data, and also, mainly, because we had to restrict the analysis to gene exons present in the intersection of the five different kits used by the different exome sequencing platforms (see Supplemental Methods).

More generally, several elements reduced the power of our mutation burden tests. First, our exome data were technically heterogeneous because DNA sequencing was performed at different places and times. Five different DNA capture kits were used, so we had to restrict our analysis to the variants included in the intersection of the five methods, which decreased the total number of variants by a factor two. Second, both in the exome and in the panel cohorts, we removed all the variants identified as monogenic causes of PM. This concerned a limited number of patients, nine out of 47 exome patients and eight out of 64 panel patients, since a number of patients with PM referred to our two genetic centers had been previously diagnosed with *ASPM* or *WDR62* biallelic pathogenic variants by Sanger sequencing and were hence not further tested in our panel nor exome cohort. But even if removed from the count, these variants identified as monogenic causes could also have taken part in a digenic (triallelic) or even oligogenic inheritance. Third, the number of patients included in each group was limited because PM is a rare phenotype.

Taken together, our data strongly suggest that clinically ascertained PM probands harbor a burden of variants in pathway-specific

PM-related genes, either in addition to a recognized Mendelian cause or in a more complex combination. Our findings validate in human patients the experimental genetic interactions observed in mice (Jayaraman et al., 2016). This validation is important because a very large brain is an essentially human feature and PM is an essentially human phenotype, difficult to modelize in animals.

New machine-learning methods, like the variant combination pathogenicity predictor (Papadimitriou et al., 2019), which predicts the pathogenicity of any bilocus variant combination using a variant list from a single individual, might be used to re-examine data from unsolved PM cases in search of variants in two genes known or suspected to interact. Putative interactions could then be validated in zebrafish.

In conclusion, we present the first study bringing to light the complex background of the apparent Mendelian phenotype of human PM, delineating centrosomal, and non-centrosomal pathways, and show an efficient way to validate digenic interactions in PM using genome-edited zebrafish.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. All variants reported in this manuscript have been submitted to the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>; ClinVar accessions SCV000998479-SCV000998508).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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