ORIGINAL ARTICLE

Proteome changes in autosomal recessive primary microcephaly

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Abstract

Background/aim: : Autosomal recessive primary microcephaly (MCPH) is a rare and genetically heterogeneous group of disorders characterized by intellectual disability and microcephaly at birth, classically without further organ involvement. MCPH3 is caused by biallelic variants in the cyclin-dependent kinase 5 regulatory subunit-associated protein 2 gene *CDK5RAP2*. In the corresponding *Cdk5rap2* mutant or Hertwig's anemia mouse model, congenital microcephaly as well as defects in the hematopoietic system, germ cells and eyes have been reported. The reduction in brain volume, particularly affecting gray matter, has been attributed mainly to disturbances in the proliferation and survival of early neuronal progenitors. In addition, defects in dendritic development and synaptogenesis exist that affect the excitation-inhibition balance. Here, we studied proteomic changes in cerebral cortices of *Cdk5rap2* mutant mice.

Material and methods: : We used large-gel two-dimensional gel (2-DE) electrophoresis to separate cortical proteins. 2-DE gels were visualized by a trained observer on a light box. Spot changes were considered with respect to presence/absence, quantitative variation and altered mobility.

Result: : We identified a reduction in more than 30 proteins that play a role in processes such as cell cytoskeleton dynamics, cell cycle progression, ciliary functions and apoptosis. These proteome changes in the MCPH3 model can be associated with various functional and morphological alterations of the developing brain.

Conclusion: : Our results shed light on potential protein candidates for the disease-associated phenotype reported in MCPH3.

1 | INTRODUCTION

Microcephaly is defined as a significant reduction of the head circumference below the mean and is highly associated with neurologic morbidity including cognitive dysfunction (Jean et al., 2020; Subramanian et al., 2019; Woods et al., 2005). Microcephaly can be acquired or hereditary in nature. One of the prototypes to study congenital microcephaly is autosomal recessive primary microcephaly (MCPH). MCPH is a group of genetically heterogeneous diseases characterized by pronounced microcephaly at birth and intellectual disability, but classically without further organ involvement (Kaindl et al., 2010, 2014; Zaqout et al., 2017). To date, variations in 28 genes have been reported to be associated with MCPH (Zaqout & Kaindl, 2022).

MCPH subtype 3 or MCPH3 is caused by biallelic variants in the cyclin-dependent kinase 5 regulatory subunit-associated protein 2 gene *CDK5RAP2* (Bond et al., 2005; Hassan et al., 2007; Issa et al., 2013; Kraemer et al., 2011). The centrosomal protein CDKRAP2 is highly expressed in the central nervous system, particularly in neural progenitors, and plays a major role in maintaining centriole engagement and unity, hence, restricting centriole replication (Barrera et al., 2010; Issa et al., 2013). It also regulates microtubule nucleation and chromosome segregation in neural progenitors (Buchman et al., 2010; Lizarraga et al., 2010). As a result, impairments in the functions of this protein cause amplification of the centriole with a predominance of the unpaired centrioles and lead to a depletion of neural progenitors due to proliferative and survival defects (Barrera et al., 2010; Buchman et al., 2010; Lizarraga et al., 2010; Kraemer et al., 2015). Furthermore, *CDK5RAP2* encodes for a regulator of CDK5 action in the Golgi apparatus of the cerebral cortex (Wang et al., 2010).

The prevailing model for the microcephaly phenotype in MCPH invokes a premature shift from symmetric to asymmetric neural progenitor-cell divisions with a subsequent depletion of the progenitor pool and a reduction of the final number of neurons. In addition, we and others have proposed a reduction in progenitor survival, abnormal dendritic development and synaptogenesis affecting the excitation-inhibition balance (Kaindl et al., 2010; Zaqout et al., 2017, 2019).

Cdk5rap2 mutant or Hertwig's anemia mice (*an/an*) are microcephalic at birth and represent an acknowledged MCPH3 mouse model for the study of MCPH pathomechanisms (Lizarraga et al., 2010; Zaqout et al., 2017, 2019, 2020). The present study complements previous research in the field of MCPH and aims to identify candidate proteins that may play a role in the pathomechanism underlying the cerebral MCPH phenotype.

2 | MATERIALS AND METHODS

2.1 | Mice

We used *Cdk5rap2* mutant or Hertwig's anemia mice (*an/an*) as a model for MCPH3 (Lizarraga et al., 2010; Zaqout et al., 2017, 2019, 2020). These mice carry an inversion of *Cdk5rap2* exon 4 that results in exon 4 skipping and the deletion of a large part of the γ-tubulin ring complex (γ TuRC) binding domain (Lizarraga et al., 2010). *an/an* mice were generated by crossing heterozygous (*+/an*) mice (*C57BL/6* background; Jackson lab, stock no. 002306). Genotyping was performed using the following PCR primers: (+/+) F 5′-TC ACT GAG CTG AAG AAG GAG AA-3′, R 5′-TGT CTT TCT GCC CTG ACA GT-3′ and (*an/an*) F 5′-GC AAT CAC TAA AAT GTC CGA TT-3′, R 5′-TGT CTT TCT GCC CTG ACA GT-3′. Mouse experimental studies have been approved by Charité-Universitätsmedizin ethics committee (registration no. T0309/09), as reported previously (Zaqout et al., 2017, 2019, 2020).

2.2 | Tissue collection and protein extraction from cerebral cortices

Cerebral cortices from postnatal day 0 (P0) *an/an* and wild-type (+/+) mice (*n* = 6 per group) were rapidly collected, pooled, snap-frozen in liquid nitrogen and stored at −80°C. Total protein was extracted as previously described with a few modifications (Kaindl et al., 2006, 2007; Klose et al., 2002; Palacino et al., 2004). Briefly, extraction was performed as follows: brain tissues were lyophilized for 24 h then homogenized by adding 1.6 parts v/w of lysis buffer 1 (0.11 M CHAPS, 50 mM TRIZMA® Base (Sigma-Aldrich, Steinheim, Germany), 50 mM KCl, and 20% w/v glycerol at pH 7.5), 0.08 parts of protease inhibitor solution I (1 Complete ™ tablet 'Roche Applied Science, Mannheim, Germany' dissolved in 2 ml of buffer 1), and 0.02 parts of protease inhibitor solution II (1.4 mM pepstatin A and 1 mM PMSF in ethanol). The samples were then cryo-grinded in the presence of liquid nitrogen.

The tissue homogenate was transferred into a 2 ml safe lock tube, then shortly thawed, and supplied with an average number of 11 glass beads (0.034 U of glass beads per combined weight of tissue, buffers and inhibitors in mg). The lysates were sonicated, with each sample sonicated six times (one cycle of 15 s each) in an ice-cold water bath, with intervals of 1.45 min in between. The obtained homogenate was stirred for 30 min in the presence of 0.025 parts v/w benzonase (Merck, Darmstadt,Germany) and 0.021 parts v/w 5 mM magnesium chloride in buffer 1 without CHAPS at 47°C. Thereafter, 6.5 M urea as well as 2 M thiourea were added, and then subjected to stirring for 30 min at room temperature (RT) until both were completely dissolved/homogenized. The protein extract was supplied with a reducing agent of 70 mM dithiothreitol, DTT (Bio-Rad, Munich, Germany), 2% v/w of ampholyte mixture Servalyte pH 2−4 (Serva, Heidelberg, Germany), corrected by the amount of urea added (Kaindl et al., 2007) and saved at −80°C until use. Protein concentration of each sample was measured using the BioRad DC Protein Assay kit based on the protocol supplied by the manufacturer (BioRad, Munich, Germany).

2.3 | Two-dimensional gel electrophoresis

The separation of brain proteins was performed using a large-gel two-dimensional gel electrophoresis (2-DE) as described previously (Kaindl et al., 2006, 2007; Klose et al., 2002; Palacino et al., 2004). The gel format was 40 cm (IEF 630 cm [SDS-PAGE] 60.75 mm [width]). Utilizing the ampholyte approach for isoelectric focusing (IEF) that represents a first dimension, a 6 ml (20 mg/ml) protein extract

of each sample and a carrier ampholyte mixture (pH 3−10) were applied to the anodic end of an IEF gel. IEF procedure was set up in vertical rod gels, where the components and running conditions for IEF have been described in detail by Klose (1999). Briefly, gels were run for 1 h at 100 V, 1 h at 300 V, 23 h at 1000 V, 30 minutes at 1500 V and lastly 10 min at 2000 V. After that, proteins were visualized in SDS-PAGE (second dimension) polyacrylamide gels through high sensitivity silver staining (Klose et al., 2002, 2006, 2007). Proteomweaver™ imaging software version 2.1 (Definiens, Munich, Germany) was used to count all visualized protein spots within all representative 2-DE brain protein patterns.

2-DE gels were visualized by a trained observer on a light box (Biotec-Fischer, Reiskirchen, Germany). Spot changes were considered with respect to presence/absence, quantitative variation and altered mobility. Mobility variants are spots that 'migrate' to a different position in the 2-DE gel referring a shift of pI and/or molecular weight (Mw). The quantitative alterations greater than 20% were selected for further analysis. Protein spots found to be reproducibly altered were further evaluated with Proteomweaver™ imaging software version 2.1 (Definiens). Following automatic spot detection and gel normalization, spot matching between all gels was monitored and edited where appropriate. The automatic normalization was applied on spot intensities to adjust them in a way generating a comparable pattern between spot pairs/different gels. The relative intensity of individual spots in 2-DE gels of *an/an* and +/+ mice was quantified using spot volumes of 16-bit gray scale images. The relative intensities of selected protein spots were measured via densitometric measurements. The student's *t*-test was utilized to display whether there is a significant difference between sample pairs ($p < 0.05$).

2.4 | Protein identification

Note that 40 ml (20 mg/ml) protein extract was separated using 1.5 mm diameter IEF and 1.0 mm (width) SDS-PAGE gels, and resulting 2-DE gels were stained following a MS compatible silver staining protocol (Kaindl et al., 2007; Shevchenko et al., 1996). The selected protein spots were excised out of 2-DE gels, and then in-gel digestion was performed by using trypsin. The resulting peptides out of tryptic digestion were analyzed by LC-MS/MS on an LCQ Deca XP IT instrument (Thermo Finnigan, Waltham, MA), where LC was coupled to ESI-MS analysis. Protein spot eluates of 15 ml were loaded onto a trapping column PepMap C18 Nano-Precolumn (5 mm, 100 Å, 300 mm id61 mm; LC Packings, Amsterdam, Netherlands) using 0.1% v/v trifluoro-acetic acid TFA at a flow rate of 20 ml/min.

Peptides were eluted onto the separation column PepMap C18 100 column (length 75 mm id615 cm; LC Packings). The elution gradient was produced upon mixing 0.1% v/v formic acid FA in water (solvent A) and 0.1% v/v FA in ACN (solvent B), and run at a flow rate of 200 nl/min. The gradient was initially increased from 5% v/v solvent B up to 50% v/v solvent B after 40 min. ESI-MS data acquisition was applied throughout the LC run. Three scan events, (i) full MS scan, (ii) zooming scan of most intense ion in full scan and (iii) MS/MS scan of the most intense ion in full scan were applied sequentially. The isolation width of precursor ions was set to 4.50 m/z, normalized collision energy at 35%, signal cut-off at 106,104, zoom scan mass width low/high at 5.00 m/z. Dynamic exclusion was permitted, exclusion mass width low/high was set at 3.00 m/z. LC-MS raw data out of MS instrument were extracted by the TurboSEQUEST algorithm as DTA files and converted to MASCOT generic format files (MGF). Mass spectra analysis was done utilizing our MASCOT in-house license with automatic searches in NCBI databases. MS/MS ion search was performed using the following parameters: (i) taxonomy: mammalia, (ii) proteolytic enzyme: trypsin, (iii) one missed cleavage was allowed, (iv) mass value of monoisotopic peak, (v) peptide 'precursor' mass tolerance was set to 1 Da, fragment mass tolerance was set to 1 Da and (vi) methionine oxidation as well as acrylamide adducts (propionamide) on cysteine residues as variable modifications, and a carbamidomethylation on cysteine residues as a fixed modification. Proteins were accepted to be correctly identified with scores of $p < 0.01$, if at least three unique peptides were identified.

3 | RESULTS AND DISCUSSION

The current study illustrates cerebrocortical proteomic profile changes in *an/an* versus +/+ mice at birth (P0). At this time point the microcephaly phenotype is already present in *an/an* mice. Our findings indicate various biological processes affected in *an/an* mice neocortices, ranging from cell division and cell death to the control of axonal arborization and dendritic growth. The modulated proteins are potential candidates for the MCPH3-associated phenotype.

3.1 | Proteomic changes in an/an cerebral cortices

To identify proteome changes associated with microcephaly in *an/an* versus +/+ P0 mice, total protein extracts from neocortical samples were separated by large-gel 2-DE (Figure S1). Protein spot pattern comparison between all representative 2-DE protein patterns of *an/an* versus +/+ mice and subsequent mass spectrometry resulted in the identification of 32 neocortical proteins downregulated and 11 proteins upregulated in *an/an* neocortices (Figure 1 and Table 1). These proteins can be functionally classified into the following categories: (i) proteins affecting the cytoskeleton, (ii) proteins affecting cell cycle and cilia, (iii) proteins affecting apoptosis and/or metabolic enzymes and oxidative stress and (iv) proteins involved in synapse function/vesicular transport. Indeed, different protein classes are linked to various biological cellular events including mRNAs processes amongst others (Table 1). Consequently, it is suggested that these proteomic changes could be directly or indirectly associated with the phenotypic changes in *an/an* not only in brain (Lizarraga et al., 2010; Zaqout et al., 2019), but also in gonad (Zaqout et al., 2017), eye (Zaqout et al., 2020) and blood (Russell et al., 1985). In this study, we focused on the cerebral cortex as it is the main affected part in the microcephaly phenotype (Kaindl et al., 2010; Woods et al., 2005).

3.2 | Proteins affecting the cytoskeleton

An increasing number of brain malformations has been associated with variations in tubulin genes. These include malformations of cortical development such as various degrees of gyral disorganization, focal or diffuse polymicrogyria, lissencephaly, hypoplasia of cranial nerves and dysmorphisms of the hindbrain (Romaniello et al., 2015). We found several proteins affecting cellular cytoskeleton organization to be downregulated in *an/an* (red nodes in Figure 1a; Table 1). Among these are microtubule-associated protein RP/EB family member 1 and 3 (Mapre1 and Mapre3). Mapre1 (Eb1) plays an important role during interphase, during which it is localized to the growing ends of the microtubules (Tirnauer et al., 2002; Zanic et al., 2009). Furthermore, it is linked to mitotic division in brain cells due to its association with the centrosomes and spindle microtubules (van de Willige et al., 2016). Mapre3 (Eb3) regulates the dynamics of the microtubule cytoskeleton by binding to the plus end of microtubules and promotes microtubule growth (Komarova et al., 2009). Both Mapre1 and Mapre3 are involved in spindle function via stabilizing microtubules by anchoring them at the centrosomes (Askham et al., 2002). It has been shown that CDK5RAP2 in human cell lines interacts with EB1 and plays a major role in the dynamics and stability of microtubules (Fong et al., 2009). Furthermore, mitotic spindle orientation has been altered in *an/an* and following *Cdk5rap2* knockdown, which can eventually affect neural progenitor's cell fate (Buchman et al., 2010; Buchman & Tsai, 2007; Lizarraga et al., 2010).
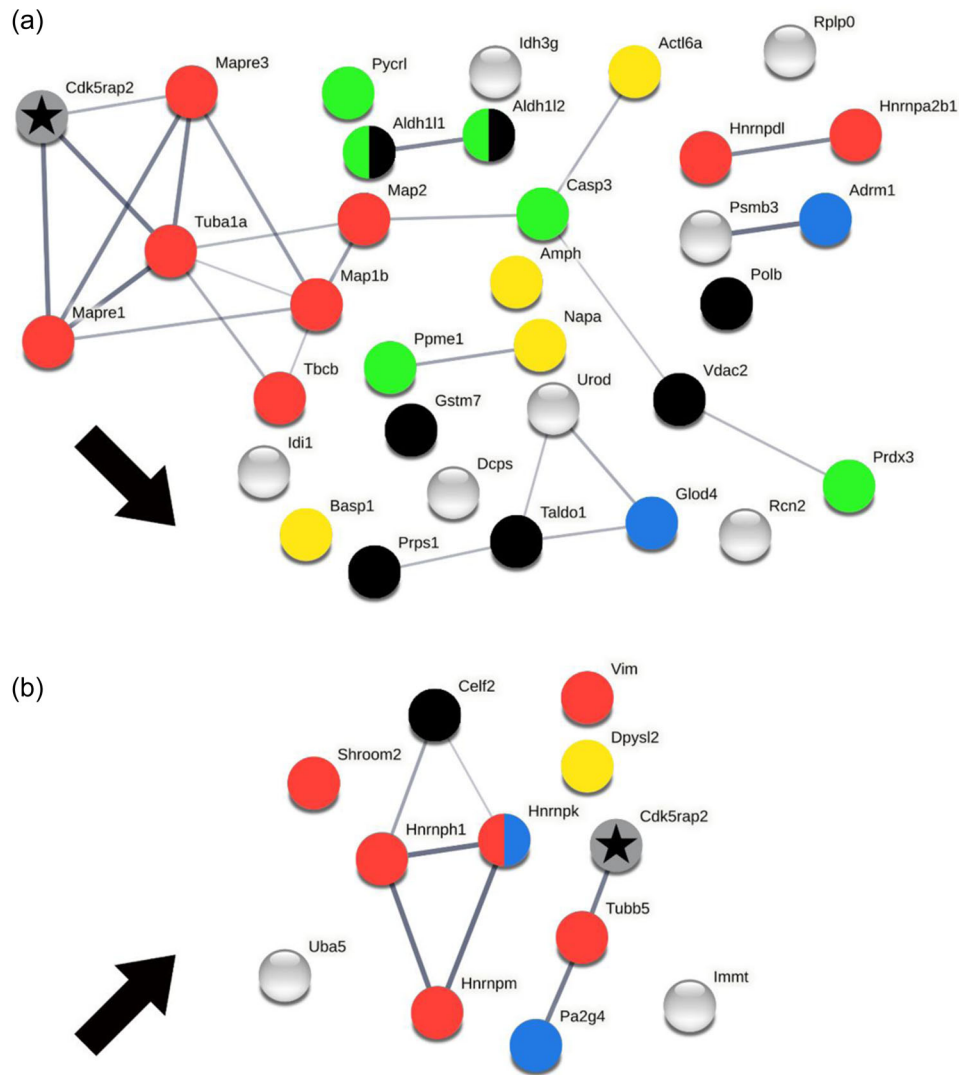
FIGURE 1 Protein-protein interactions for the down- (a) and up- (b) regulated proteins in P0 *an/an* cerebral cortex. In the network, proteins are represented as nodes and the line thickness indicates the strength of data support. Nodes' colors represent the following protein classes: red (proteins affecting the cytoskeleton), blue (proteins affecting cell cycle), green (proteins affecting apoptosis and/or oxidative stress), black (metabolic enzymes) and yellow (proteins involved in synapse function/vesicular transport). Network generated by STRING software {https://string-db.org/}

Microtubule-associated protein 1B (Map1b) is also found to be downregulated in *an/an*. In neurons and axons, Map1b and phosphorylated Map1b maintain a dynamic balance between cytoskeletal components (Yang et al., 2012). They also regulate the stability and interaction of microtubules as well as actin to promote axonal growth, neural connectivity, and regeneration in the central nervous system (Yang et al., 2012). Tubulin alpha-1A chain protein (Tuba1a) is also downregulated in *an/an*. It has been reported that a missense variation in exon 4 of *Tuba1a* gene in a hyperactive N-ethyl-N-nitrosourea induced mouse mutant with abnormal lamination of the hippocampus (Poirier et al., 2007). Retrospective examination of MRI images suggests that patients with *TUBA1A*

variations share not only cortical dysgenesis, but also cerebellar, hippocampal, corpus callosum, and brainstem abnormalities (Poirier et al., 2007). In addition, Tubulin-folding cofactor B (Tbcb) is downregulated in *an/an*. Tbcb is localized at spindle and midzone microtubules during mitosis and is also involved in microtubule dynamics and plasticity (Carranza et al., 2013; Lopez-Fanarraga et al., 2007). Collectively, the proteins of the tubulin family are involved in microtubule assembly, which is an essential step in neurogenesis (Lasser et al., 2018; Yang et al., 2012). Indeed, the tubulin gene family is mainly expressed in postmitotic neurons during cortical development with a specific spatial and temporal expression pattern (Romaniello et al., 2015). Thus, downregulation of

TABLE 1 Brain protein alterations in P0 *an/an* versus +/+ neocortices

Accession number#	Protein Name	Quantitative changes (fold change)***
Proteins affecting the cytoskeleton (red nodes in Figure 1)		
NP_031922	Microtubule-associated protein RP/EB family member 1 (Mapre1)	▼1.4-fold* P = 0.024
NP_579928	Microtubule-associated protein RP/EB family member 3 (Mapre3)	▼1.4-fold* P = 0.034
NP_032660	Microtubule-associated protein 1B (Map1b)	▼1.3-fold* P = 0.014
AAI50946	Microtubule-associated protein 2 (Map2)	▼2-fold* P = 0.040
NP_035783	Tubulin alpha-1A chain (Tuba1a)	▼1.3-fold* P = 0.011
AAH10684	Tubulin-folding cofactor B (Tbcb)	▼1.4-fold** P = 0.008
NP_035785	Tubulin beta-5 chain (Tubb5)	▲1.3-fold* P = 0.043
NP_057899	Heterogeneous nuclear ribonucleoprotein D-like (Hnrnpdl)	▼1.4-fold* P = 0.047
NP_058086	Heterogeneous nuclear ribonucleoproteins A2/B1 (Hnrnpa2b1)	▼1.4-fold** P = 0.001
NP_001334416	Heterogeneous nuclear ribonucleoprotein H (Hnrnph1)	▲1.2-fold* P = 0.048
NP_084080	Heterogeneous nuclear ribonucleoprotein M (Hnrnpm)	▲1.3-fold** P = 0.009
ACC69193	Heterogeneous nuclear ribonucleoprotein K (Hnrnpk)	▲1.4-fold* P = 0.023
NP_035831	Vimentin (Vim)	▲1.3-fold* P = 0.043
NP_766029	Protein Shroom2 (Shroom2)	▲1.3-fold* P = 0.043
Proteins affecting cell cycle (blue nodes in Figure 1)		
NP_080305	Glyoxalase domain-containing protein 4 (Glod4)	▼1.2-fold* P = 0.047
NP_062796	Proteasomal ubiquitin receptor ADRM1 (Adrm1)	▼1.4-fold** P = 0.007
NP_035249	Proliferation-associated protein 2G4 (Pa2g4)	▲1.4-fold*** P = 0.004
ACC69193	Heterogeneous nuclear ribonucleoprotein K (Hnrnpk)	▲1.4-fold* P = 0.023
Proteins affecting apoptosis and/or oxidative stress (green nodes in Figure 1)		
NP_001011993	Pyrroline-5-carboxylate reductase 3 (Pycrl)	▼2-fold** P = 0.009
NP_031478	Thioredoxin-dependent peroxide reductase, mitochondrial (Prdx3)	▼1.3-fold* P = 0.037
NP_082568	Protein phosphatase methylesterase 1 (Ppme1)	▼1.3-fold* P = 0.012
NP_001271338	Caspase-3 (Casp3)	▼1.2-fold* P = 0.044
NP_001343341	Cytosolic 10-formyltetrahydrofolate dehydrogenase (Aldh1l1)	▼1.4-fold* P = 0.013
NP_705771	Mitochondrial 10-formyltetrahydrofolate dehydrogenase (Aldh1l2)	▼1.4-fold** P = 0.013
Proteins affecting metabolic enzymes (black nodes in Figure 1)		
NP_080948	Glutathione S-transferase Mu 7 (Gstm7)	▼1.4-fold* P = 0.033
NP_001343341	Cytosolic 10-formyltetrahydrofolate dehydrogenase (Aldh1l1)	▼1.4-fold * P = 0.013
NP_705771	Mitochondrial 10-formyltetrahydrofolate dehydrogenase (Aldh1l2)	▼1.4-fold ** P = 0.013
NP_032643	Malate dehydrogenase, mitochondrial (Mdh2)	▼1.3-fold*** P = 0.001
NP_035658	Transaldolase (Taldo1)	▼1.4-fold* P = 0.042
NP_067438	Ribose-phosphate pyrophosphokinase 1 (Prps1)	▼1.4-fold* P = 0.023
NP_035260	DNA polymerase beta (Polb)	▼1.2-fold* P = 0.036
NP_035825	Voltage-dependent anion-selective channel protein 2 (Vdac2)	▼1.2-fold* P = 0.044
NP_059064	CUGBP Elav-like family member 2 (Celf2)	▲1.4-fold* P = 0.016
Proteins involved in synapse function/vesicular transport (yellow nodes in Figure 1)		
EDL32705	Amphiphysin (Amph)	▼2-fold* P = 0.040
NP_080174	Alpha-soluble NSF attachment protein (Napa)	▼1.3-fold* P = 0.011
NP_062647	Actin-like protein 6A (Actl6a)	▼1.4-fold * P = 0.011
NP_081671	Brain acid soluble protein 1 (Basp1)	▼1.2-fold* P = 0.049
NP_034085	Dihydropyrimidinase-related protein 2 (Dpysl2)	▲1.6-fold** P = 0.001
Other proteins		
XP_021062826	m7GpppX diphosphatase (DCPS)	▼1.4-fold* P = 0.047
NP_033504	Uroporphyrinogen decarboxylase (Urod)	▼1.4-fold* P = 0.047

(Continues)

TABLE 1 (Continued)

AAB19006	Carbonyl reductase [NADPH] 1	▼1.2-fold* P = 0.025
NP_032349	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial (Idh3g)	▼1.2-fold* P = 0.025
NP_031501	60S acidic ribosomal protein P0 (Rplp0)	▼1.4-fold* P = 0.042
NP_036101	Proteasome subunit beta type-3 (Psmb3)	▼1.3-fold* P = 0.037
NP_036122	Reticulocalbin-2 (Rcn2)	▼1.2-fold* P = 0.049
NP_663335	Isopentenyl-diphosphate Delta-isomerase 1 (Idi1)	▼1.3-fold* P = 0.040
NP_079968	Ubiquitin-like modifier-activating enzyme 5 (Uba5)	▲1.3-fold* P = 0.043
NP_083949	MICOS complex subunit Mic60 (Immt)	▲1.6-fold** P = 0.001

Note: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

several members of this protein family in *an/an* may contribute to the neuronal differentiation defects described in our previous study (Zaqout et al., 2019).

Heterogeneous nuclear ribonucleoproteins (Hnrnps) play a key role in the regulation of alternative splicing (Bekenstein & Soreq, 2013). Hnrnps accompany transcripts from stages of transcriptional regulation through splicing and posttranscriptional regulation and affect the majority of expressed genes in mammals (Bekenstein & Soreq, 2013; Kim et al., 2013). It has been suggested that defects in these genes are correlated with the severity of symptoms in several neurodegenerative diseases, including Alzheimer's disease, spinal muscular atrophy, fronto-temporal lobar degeneration, amyotrophic lateral sclerosis, multiple sclerosis, hereditary spastic paraparesis and HTLV-I associated myelopathy/tropical spastic paraparesis (Low et al., 2021). It has also been described that cell cycle, cytoskeletal rearrangement and transcriptional regulation were all affected upon depletion of Hnrnps (Yeh et al., 2014). Our findings show that both Hnrnpdl and Hnrnpa2/b1 are downregulated while Hnrnph1, Hnrnpm and Hnrnpk are upregulated in *an/an* (Figure 1 and Table 1). Therefore, it is unsurprising that Hnrnps dysfunctions can have an impact in microcephaly conditions.

Vimentin (Vim) is an intermediate filament that plays a role in maintaining cellular shape and stabilizing cytoskeletal interaction mainly in epithelial and glial cells (Schnitzer et al., 1981). On the other hand, the protein Shroom2 contributes to the formation of a contractile network within epithelial cells (Lee et al., 2009). Both Vim and Shroom2 are upregulated in *an/an* (Figure 1b and Table 1).

3.3 | Proteins affecting cell cycle

Proteomic analyses in *an/an* mice identified neocortical proteins that play a crucial role in the cell cycle (blue nodes in Figure 1 and Table 1). Proteasomal ubiquitin receptor protein (Adrm1) is important for protein homeostasis maintenance by removing misfolded and damaged proteins (Shenoy, 2015). It also plays a role in cell proliferation and, if downregulated, causes cell cycle arrest in the G0/G1 phase (Shenoy, 2015). Downregulation of Adrm1 in *an/an* can affect cellular functions due to the inability to remove unnecessary proteins, which could interfere with the normal cell cycle progression. Indeed, it has been shown that Cdk5rap2 plays an important role in maintaining cell cycle progression and controlling cell cycle exit during early neurogenesis (Buchman et al., 2010; Lizarraga et al., 2010; Zaqout et al., 2017). Taken together, downregulation of this group of proteins in our present study can have an important impact on cell proliferation and cell cycle progression in *an/an*. Moreover, it has been shown that CDK5RAP2 regulates centriole maturation and cilia assembly (Barrera et al., 2010). This can raise further investigations to detect potential ciliopathies in *an/an* mice and other conditions associated with microcephaly.

Glyoxalase domain-containing protein 4 (Glod4) is not only involved in cell cycle control, but also in cilia assembly, cilia length control, basal body/centriole numbers and the distance between basal bodies/centrioles (Albee et al., 2013). Hence, downregulation of Glod4 in *an/an* can affect the normal cell progression and lead to ciliopathies. This is in line with the fact that other MCPH proteins are implicated in ciliogenesis and cilia stability during brain development (Cuenca et al., 2019; Ding et al., 2019; Farooq et al., 2020).

Proliferation-associated protein 2G4 (Pa2g4) is found to be upregulated in *an/an* (Figure 1b and Table 1). It is worth noting that Pa2g4 is present in preribosomal ribonucleoprotein complexes and is involved in cellular growth and differentiation (Kowalinski et al., 2007; Radomski & Jost, 1995). Similarly, Hnrnpk is also upregulated in *an/an* (Figure 1b and Table 1). Hnrnpk has been reported to play an important role during cell cycle and in multiple cytoskeletal-related mRNA processes required for axon outgrowth (Hutchins et al., 2015; Barboro et al., 2014). Upregulation of Pa2g4 and Hnrnpk might be due to functional redundancy to compensate for the deficiency of other proteins in the biological system of the *an/an* mice.

3.4 | Proteins affecting apoptosis and/or oxidative stress

Some of the downregulated cortical proteins detected in *an/an* are involved in apoptosis (green nodes in Figure 1 and Table 1). The loss of pyroline-5-carboxylate reductase (Pycrl) function can lead to a reduction in the mitochondrial membrane potential and an increased susceptibility to apoptosis due to oxidative stress (Nakayama et al., 2015). Variations in the *PYCRL* gene in humans have been linked to postnatal microcephaly, hypomyelination and reduction in white matter volume (Nakayama et al., 2015). Likewise, the depletion of mitochondrial thioredoxin-dependent peroxide reductase (Prdx3) can lead to oxidative damages and apoptosis (De Simoni et al., 2008). In addition, the nuclear protein phosphatase methylesterase 1 (Ppme1) is downregulated in *an/an*. The knockdown of PPME1 in human cancer cell lines has been reported to inhibit cell proliferation and induce cell apoptosis (Li et al., 2014).

Surprisingly, cysteine-aspartic acid protease 3 (Casp3) and mitochondrial 10-formyltetrahydrofolate dehydrogenase (Aldh1l1 and Aldh1l2) are downregulated in *an/an* (Figure 1a and Table 1). Casp3 is a well-known product of the sequential activation of caspases, which plays a key role in the execution phase of cell apoptosis (Yakovlev et al., 2010). Others and we detected a massive increase in the number of Casp3 positive apoptotic cells during early neurogenesis (E12.5) in *an/an* (Lizarraga et al., 2010). Controversially, *Cdk5rap2* knockdown experiments conducted in mice has not been associated with increased apoptosis (Buchman et al., 2010). The downregulation of Casp3 protein in our present study on P0 *an/an* mice can be partially explained by the fact that the cortical samples were taken from significantly older mice, that these older mice had significantly lower Casp3 levels and that premature neuronal differentiation in *an/an* can be associated with lower Casp3 levels at birth (Yakovlev et al., 2010). Likewise, both Aldh1l1 and Aldh1l2 are downregulated in P0 *an/an* mice pointing towards less apoptosis at this developmental stage (Krupenko & Horita, 2019).

3.5 | Proteins affecting metabolic enzymes

Downregulation of several metabolic enzymes have been detected in *an/an* (black nodes in Figure 1, and Table 1). In addition to their role in apoptosis, both Aldh1l1 and Aldh1l2 control folate metabolism by regulating the distribution of 1-carbon groups in the cytosol and mitochondria (Krupenko & Krupenko, 2018). Aldh1l1 belongs to the aldehyde dehydrogenase family and is tightly regulated during brain development and (Anthony & Heintz, 2007;

Cahoy et al., 2008). In fact, folates are very important for DNA biosynthesis, cellular proliferation and amino acid metabolism (Stark et al., 2021; Tedeschi et al., 2013). Consequently, folate deficiency leads to severe neurological deficits including spina bifida and anencephaly (Boot et al., 2003; Steele et al., 2020). Hence, the use of folate supplementations at early gestational ages reduces the rate of these defects (de la Fournière et al., 2020; Ren, 2015). In addition, folates play an important role in the biosynthesis of cellular cytoskeleton (Stark et al., 2021). Following carrier-mediated uptake, folates are polyglutamylated by folylpoly-γ-glutamate synthetase (FPGS), resulting in their intracellular retention. It was shown that cFPGS is a cytoskeleton-microtubule associated protein and is associated with the insoluble cellular fraction involving cytoskeleton and membranes (Stark et al., 2021). On the other hand, Aldh1l1 is also robustly expressed in astrocytes, and this may be correlated with role of Cdk5rap2 in regulating morphological differentiation of astrocytes (Beyer et al., 2021; Cahoy et al., 2008; Kang et al., 2020).

Other downregulated metabolic enzymes include mitochondrial malate dehydrogenase (Mdh2), transaldolase (Taldo1), ribose-phosphate pyrophosphokinase 1 (Prps1) and DNA polymerase beta (Polb). First, Mdh2 plays a role in energy and cellular metabolism by catalyzing the oxidation reaction of malate to oxaloacetate within the citric acid cycle (Takahashi-Íñiguez et al., 2016). It has been shown that MDH2 knockdown reduce cell proliferation in prostate cancer cell lines (Liu et al., 2013). Second, Taldo1 is a major enzyme involved in the pentose phosphate pathway (Perl et al., 2011). In human, TALDO1 deficiency is a rare condition presents with growth retardation, dysmorphic features, congenital heart disease, progressive nodular liver fibrosis, pancytopenia and bleeding tendency (Eyaid et al., 2013; Tylki-Szymanska et al., 2014). Third, Prps1 is responsible for the catalyzation of the ribose 5-phosphate to 5-phosphoribosyl-1-pyrophosphate which is important for metabolism of purines and nucleotides (de Brouwer et al., 2010). In addition, it has been recently shown that PRPS1 maintains the stemness of pluripotent stem cell (PSCs) and its knockout promotes PSCs differentiation and triggeres DNA damage and apoptosis (Yang et al., 2021). Human variations in *PRPS1* results in phosphoribosylpyrophosphate synthetase superactivity leading uric acid overproduction (Mittal et al., 2015). *PRPS1* individuals show various neurodevelopment abnormalities which include hypotonia, ataxia, isolated hearing loss and severe congenital encephalopathy (Mercati et al., 2020; Mittal et al., 2015). Sensorineural hearing loss has been reported in a patient with nonsense CDK5RAP2 variation (Pagnamenta et al., 2012). Finally, Polb plays an essential role in the base excision repair pathway, a process known as gap-filling DNA synthesis (Beard et al., 2006). Downreg-

ulation of *POLB* associates with neurodegeneration due to apoptosis and impaired memory and synaptic plasticity (Sykora et al., 2015). Apparently, the proliferation and survival of early neuronal progenitors are vulnerable to any changes in mitochondrial-related metabolism (Issa et al., 2013). Therefore, the aforementioned metabolic enzymes are potential candidates to investigate the direct or indirect role of mitochondria in MCPH pathomechanism.

Glutathione S-transferase Mu 7 (Gstm7) is involved in sperm function, and spermatogenic cell proliferation (Li et al., 2013). GSTm7 is one of the detoxfication enzymes in the testis that act against oxidative stress products (Wang et al., 2007). Downregulation of Gstm7 in *an/an* might contribute to a germ cell defect reported in *an/an* (Zaqout et al., 2017; Russell et al., 1985). In the brain, glutathione is a major cellular antioxidant defense mechanism by acting as a substrate for the enzyme glutathione peroxidase (Raps et al., 1989). Intriguingly, neurons are much prone to oxidative stress compared to glial cells due to lower levels of antioxidants such as glutathione (Valko et al., 2007; Dringen et al., 2000). This might explain the subtle changes in glial cells as opposed to the neuronal defects reported in *an/an* (Lizarraga et al., 2010; Zaqout et al., 2019).

The only upregulated metabolic enzyme detected in *an/an* was CUGBP Elav-like family member 2 (Celf2) which regulates pre-mRNA alternative splicing and may be involved in mRNA editing (Blech-Hermoni et al., 2013). It has been shown that Celf2—also known as Napor—is mainly expressed in subsets of differentiating neurons in the forebrain and hindbrain (Choi et al., 2003).

3.6 | Proteins involved in synapse function/vesicular transport

Some changes have also been seen in proteins responsible for synapse function and vesicular transport (yellow nodes in Figure 1, and Table 1). Most of these are downregulated in *an/an* and include amphiphysin (Amph), alpha-soluble NSF attachment protein (Napa), actin-like protein 6A (Actl6a) and brain acid soluble protein 1 (Basp1). On the other hand, dihydropyrimidinase-related protein 2 (Dpysl2) is upregulated in *an/an*. These changes might be a primary effect or a secondary consequence of excitation-inhibition imbalance reorted in *an/an* (Zaqout et al., 2019).

Amph is associated with the cytoplasmic surface of synaptic vesicles and regulates exocytosis in synapses by controlling the properties of the membrane-associated cytoskeleton (Galic et al., 2012). Amph is phosphorylated by Cyclin-dependent kinase-like 5 (Cdkl5), which is crucial for neuronal development (Sekiguchi et al.,

2013). Napa plays a role in completing synaptic membrane fusion and interacts with the putative synaptic calcium sensor protein synaptotagmin, thereby influencing calcium-regulated exocytosis (Stenbeck, 1998). It has also been suggested that Napa may be involved in vesicular transport between the endoplasmic reticulum and the Golgi apparatus (Andreeva et al., 2005). Actl6a is part of actin-related proteins (ARPs) that are generally involved in a variety of cellular processes, including vesicular transport, spindle orientation, nuclear migration, and chromatin remodeling (Schafer & Schroer, 1999). Actl6a forms a subunit, which is used in several complexes that are important for embryonic stem cell maintenance (Lu et al., 2015). Interestingly, Actl6a specifically belongs to the neural progenitors-specific chromatin remodeling complex (npBAF), which is required for the proliferation of neural progenitors (Kadoch & Crabtree, 2015). Our findings also show downregulation of Basp1, a presynaptic membrane protein involved in axon guidance, neurodegeneration and synaptic plasticity (Forsova & Zakharov, 2016). In contrast to other proteins, Dpysl2 is upregulated in *an/an* (Figure 1b and Table 1). It plays a role in synaptic signalling through interactions with calcium channels and in mediating growth cone collapse (Stratton et al., 2020).

4 | CONCLUSION

We investigated proteomic changes in *an/an* as a mouse model for MCPH3 and thus congenital microcephaly. Our findings uncover candidate proteins potentially involved in the pathogenesis of MCPH3. Alterations in enzymes involved in folate/mitochondrial metabolism and free radical scavengers such as glutathione trigger novel insights behind the etiology of MCPH. Further functional studies are necessitated to confirm these findings. This will also open the door for similar studies on other MCPH animal models and at earlier developmental stages to discover additional proteomic changes. Together this will help us to understand biological changes that potentially contribute to the pathomechanism of MCPH conditions beyond the currently prevailing models.

CONFLICTS OF INTEREST

The authors declare 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.

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