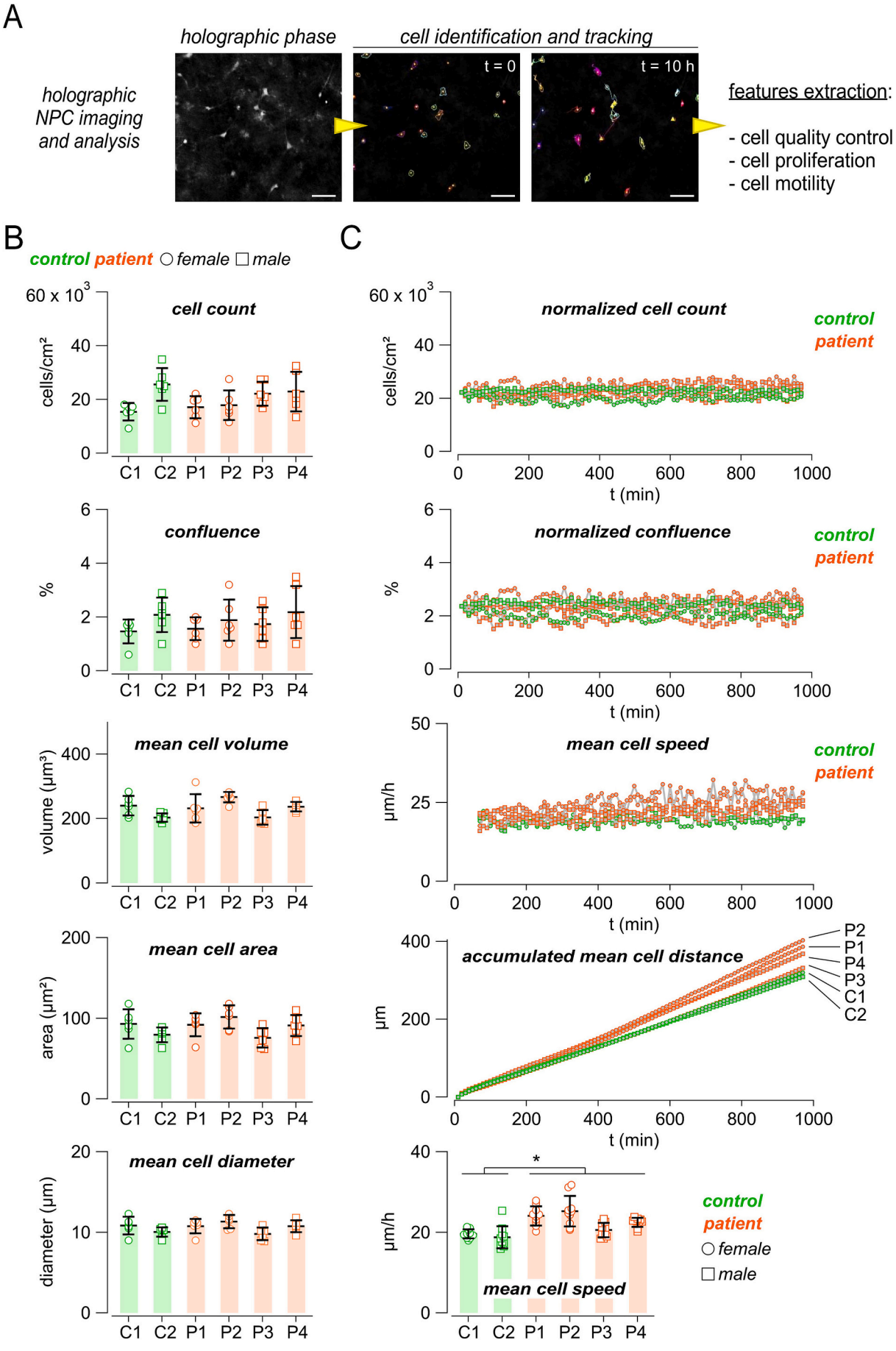

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Fig. 6. Label-free imaging of MWS iPSC-derived NPCs. Holographic live-imaging of iPSC-derived NPC revealed comparable basic morphology but increased motility in MWS-derived cells. Control and patient iPSC-derived NPCs were seeded in 24-well plates and imaged every 10 min at 20× magnification over a 16-h period using label-free holographic microscopy. (A) Representative holographic phase image before (left) and after (right) cell segmentation and tracking. Scale bar: 50 μm. (B) Summary graphs showing output variables measured after 1 h: cell count, confluence, mean cell volume, mean cell area, and mean cell diameter. Bar graphs represent mean ± S.D.; individual biological replicates are shown as dots ($n = 6$). No statistically significant differences were detected by linear mixed models. (C) Summary graphs showing dynamic output variables over the 16-h imaging period: normalized cell count and confluence (values normalized to the mean of all conditions at time 0), mean cell speed, and accumulated mean cell distance. The bottom graph shows mean cell speed averaged per cell line. Bar graphs represent mean ± S.D.; individual biological replicates are shown as dots (n = 6). Statistical analysis was performed by grouping data by condition (C1 and C2 as controls vs. P1-P4 as patient-derived lines). *, $p < 0.05$ as determined by linear mixed models ($n = 10$ replicates from 3 independent cell preparations).

(ECM) interaction in upregulated genes, while no significant enrichment was found among downregulated genes (Fig. 5D). Consistently, GO analysis showed that upregulated genes were enriched in biological processes such as vasculature development, tube morphogenesis, and system development, in cellular components like collagen-containing extracellular matrix, and in molecular functions including ECM structural constituents and DNA-binding transcription factor activity (Supplementary Fig. 1). In contrast, downregulated genes were modestly enriched in GO categories related to antigen processing, cellular response to metal ions, and endoplasmic reticulum components (Supplementary Fig. 2).

Together, these results reveal critical transcriptional dysregulation in MWS NPCs, characterized by impaired neurogenic programs and aberrant activation of extracellular matrix and non-neural developmental pathways, potentially contributing to the neuropathological and systemic features of Mowat-Wilson syndrome.

3.6. Increased cell motility in MWS NPCs and early neurons

To explore the functional consequences of transcriptional dysregulation, we performed label-free holographic live imaging of NPCs. While overall growth and morphological metrics, including cell count, confluence, mean cell volume, area, and diameter, were comparable between groups, MWS NPCs exhibited significantly increased motility, as evidenced by higher cell speed and greater accumulated distance over time (Fig. 6A-C). These findings are consistent with transcriptomic data indicating dysregulation of ECM interaction pathways and suggest that *ZEB2* dysfunction affects the dynamic behavior of neural progenitor cells.

To assess whether these alterations persisted during early neuronal differentiation, MWS and control NPCs were further differentiated into early neurons and monitored by holographic imaging over a 10-day period. Increased motility was observed in MWS-derived cells during the initial stages of differentiation (day 1), but this difference disappeared at later time points (Fig. 7A-B). Despite these early dynamic differences, immunofluorescence analysis at day 10 revealed no significant differences in the proportion of MAP2-positive neurons, nor in MAP2 signal intensity or positive area (Fig. 7C–D), indicating that early neuronal maturation is maintained in MWS cells despite altered motility behavior.

4. Discussion

This study expands our understanding of *ZEB2*-associated neurodevelopmental dysfunction by employing patient-derived iPSC models of MWS. Through transcriptomic and phenotypic analyses of neural progenitor cells (NPCs) and early neurons, we demonstrate that *ZEB2* haploinsufficiency disrupts key processes in early neural development, including radial glial maturation, neuronal specification, ECM regulation, and cell motility.

Consistent with the known role of *ZEB2* in neurodevelopment (Epifanova et al., 2019; Tomassy et al., 2013; van den Berghe et al., 2013), MWS-derived NPCs exhibited transcriptional alterations affecting both neurogenic and glial lineage programs. The significant downregulation of radial glial markers such as *NES*, *PAX6*, and *SLC1A3* suggests a defective neuroepithelial-to-radial glia transition, in line with findings from animal models (Benito-Kwiecinski et al., 2021; Epifanova et al., 2019). Moreover, other downregulated genes are critical for neurogenesis and synaptic function (e.g., *CER1*, *PCP4*, *BEND6*, *SLCO1C1*, *SPARCL1*, *CACNB2*, *DOC2B*) and some have been implicated in neurodevelopmental disorders, including *GABBR2*, *CPNE6*, *KCNN3*, and *MCHR1* (Anazi et al., 2017; Bauer et al., 2019; Dai et al., 2013; Diez et al., 2021; Friedrich et al., 2008; Friedrich et al., 2008; Gripp et al., 2021; Hou et al., 2013; Kucukdereli et al., 2011; Martins et al., 2022; Mayerl et al., 2022; Mouton-Liger et al., 2011; Mullins et al., 2021; Yoo et al., 2017). These observations support the notion that *ZEB2* dysfunction perturbs early neural lineage specification and maturation, potentially contributing to the excitation-inhibition imbalance and neurodevelopmental deficits underlying epilepsy in MWS.

Importantly, we identified substantial transcriptional upregulation of genes associated with ECM organization and non-neural lineages, including vasculature and skeletal development. Elevated expression of *COL1A1*, *MMP25*, *FBLN2*, *ANGPT2*, and *DKK2*, among others, points to aberrant activation of mesodermal and endothelial programs in neural cultures. This suggests a failure of *ZEB2*-deficient NPCs to properly repress alternative lineage trajectories, consistent with *ZEB2*'s known role in lineage restriction and epithelial-mesenchymal transition (EMT) pathways (Vandewalle et al., 2005; Ninfali et al., 2023; Charney et al., 2023; Chng et al., 2010). The upregulation of vasculature-related genes may also provide a molecular link to systemic MWS manifestations such as cardiovascular anomalies and connective tissue involvement (Epifanova et al., 2019; St Peter et al., 2024).

A particularly intriguing finding of our study is the increased motility of MWS-derived NPCs and early neurons, as revealed by label-free holographic live imaging. While previous studies in animal models have reported defective migration of *ZEB2*-deficient interneurons, our data show that MWS cells exhibit increased intrinsic motility, moving more rapidly and covering greater distances in vitro. However, motility and migration are distinct phenomena: motility refers to a cell's inherent ability to move, while migration implies directed, context-specific movement often guided by environmental cues. Our findings suggest that, although MWS cells are more motile, they may lack the necessary directional control for proper migration in vivo. This may stem from dysregulation of ECM genes, adhesion molecules, or cytoskeletal regulators that coordinate migration and positioning. Thus, *ZEB2* dysfunction may decouple intrinsic motility and functional migration in MWS, underscoring the need for future studies using spatially structured systems (e.g., microfluidic devices or 3D models) to assess directed migration during human brain development.

Interestingly, while global downregulation of neurogenic pathways was evident, the number of significantly downregulated genes was relatively small compared to the larger set of upregulated genes. This imbalance between upregulated and downregulated transcripts may reflect the dominant repressive role of *ZEB2* on non-neural genes and the secondary consequences of losing this repression during neural differentiation (Ninfali et al., 2023). Alternatively, it could indicate a compensatory response attempting to maintain neural identity in the absence of proper transcriptional cues.

In this study, molecular and functional profiling were performed by comparing all control NPC lines as a group versus all MWS lines as a group. Although our group-level analysis successfully revealed shared disease-associated signatures across multiple patient lines, it may also

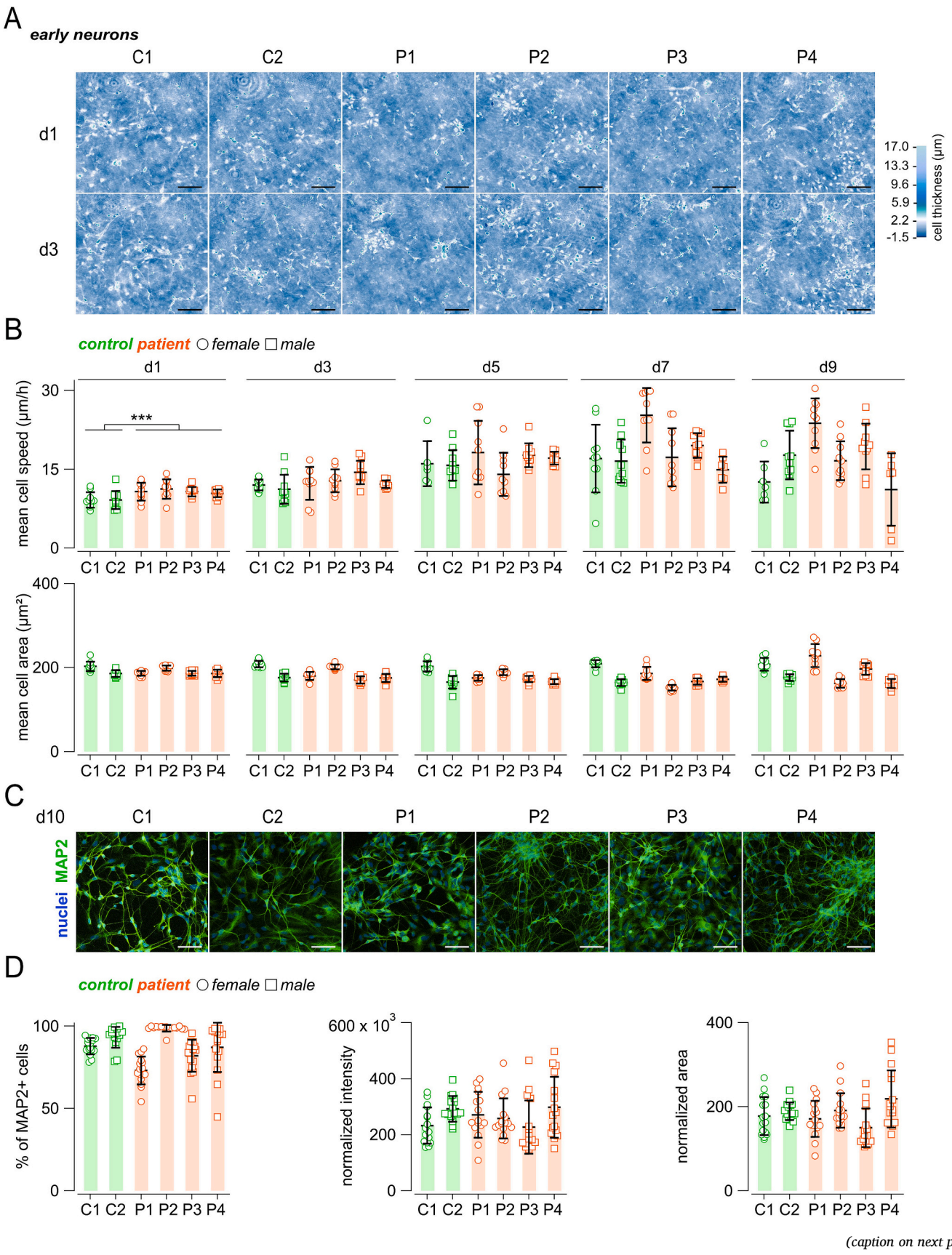

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Fig. 7. Altered motility in MWS early neurons. Control and MWS iPSC-derived NPCs were induced to differentiated into early neurons and monitored via holographic live-cell imaging for 10 days. On day 10, cells were fixed and stained for the neuronal marker MAP2. (A) Representative holographic phase images of early neurons at day 1 (d1) and day 3 (d3) for control and MWS-derived lines. Colour scale indicates cell thickness. Scale bar: 100 μm (B) Summary graphs showing mean cell speed (top) and mean cell area (bottom) at the indicated time points (d1, d3, d5, d7, and d9). Bar graphs represent mean ± S.D.; individual replicates are shown as dots (n = 10 replicates from 3 independent cell preparations). Statistical analysis was performed by grouping data by condition (C1 and C2 as controls vs. P1-P4 as patient-derived lines). ***, $p < 0.001$ as determined by linear mixed models. (C) Representative immunofluorescence images of early neurons at day 10 stained for MAP2 (green) and nuclei (blue). Scale bar: 100 μm. (D) Quantification of MAP2 expression in terms of frequency of expression (percentage of MAP2-positive cells), normalized intensity (total MAP2 signal intensity divided by the number of positive cells), and normalized area (total MPA2-positive area divided the number of positive cells). No statistically significant differences were detected (linear mixed models; $n = 16$ replicates from 4 independent cell preparations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have masked variant- or line-specific effects. For example, the GABAergic neuronal markers *SLC32A1*, *GAD1*, and *GAD2* showed reductions in three out of four MWS lines. These findings are consistent with observations from animal models and previous iPSC-based studies (Benito-Kwiecinski et al., 2021; Epifanova et al., 2019; Schuster et al., 2022), indicating that *ZEB2* dysfunction perturbs interneurons maturation. Yet, these differences did not reach statistical significance in the aggregated dataset (Fig. 4F).

A systematic per-line comparison could therefore uncover distinct transcriptional signatures associated with specific *ZEB2* mutations and reveal potential genotype–phenotype correlations. However, rigorous assessment of such effects will require larger cohorts of independent iPSC lines per genotype and/or the generation of isogenic corrected controls to disentangle true variant-driven changes from line-to-line biological or technical variability. Addressing these aspects in future work will provide a more comprehensive understanding of *ZEB2*'s diverse regulatory roles and refine our interpretation of patient-specific molecular phenotypes in MWS.

Collectively, our results indicate that *ZEB2* haploinsufficiency disrupts early human neurodevelopment through multiple, converging mechanisms: impaired lineage specification, dysregulation of neurogenic and non-neural transcriptional programs, and altered progenitor cell dynamics. These disruptions likely contribute to core clinical features of MWS, including intellectual disability, epilepsy, and brain structural anomalies such as corpus callosum hypoplasia. Future studies should extend these findings by examining later stages of neuronal maturation, synaptic function, and interneuron network activity using long-term differentiated cultures and electrophysiological analyses.

5. Conclusion

This study advances our understanding of *ZEB2*'s role in human neurodevelopment and provides a robust platform for further exploration of MWS pathogenesis. By generating and thoroughly validating high-quality iPSC lines from four unrelated MWS patients carrying distinct *ZEB2* variants, each with well-documented clinical profiles, we have established a valuable and reproducible human-based model system. These patient-specific iPSCs not only recapitulate key molecular and cellular features of the disorder but also represent a critical resource for future mechanistic investigations. Their application will enable deeper exploration of *ZEB2*-regulated transcriptional and epigenetic programs, interneuron specification, and cortical circuit formation, particularly in relation to seizure pathophysiology. Importantly, these patient-derived in vitro models also pave the way for precision medicine approaches, including genotype–phenotype correlation studies, high-throughput drug screening, and the development of targeted therapeutic strategies aimed at restoring *ZEB2* function or correcting downstream dysregulated pathways in MWS.

CRediT authorship contribution statement

Ilaria Musante: Visualization, Validation, Methodology, Investigation, Formal analysis. **Giulia Gorrieri:** Visualization, Methodology, Investigation. **Serena Tamburro:** Methodology, Investigation. **Giulia Ferrera:** Resources. **Simona Baldassari:** Methodology, Investigation. **Anna Fetta:** Resources. **Stefano Giuseppe Caraffi:** Resources, Formal analysis. **Aglaia Vignoli:** Resources. **Giovanni Fiorito:** Writing – review & editing, Data curation. **Livia Garavelli:** Resources. **Maria Paola Canevini:** Supervision, Resources. **Duccio Maria Cordelli:** Supervision, Resources. **Federico Zara:** Supervision, Resources. **Emilia Ricci:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Paolo Scudieri:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish.

na

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University of Milan (protocol number 11/23, dated 18 January 2023).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2025.107205.

Data availability

All relevant data are included in the manuscript and its supporting information files. The RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE311389. Additional information may be obtained from the corresponding author upon request.

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