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RESEARCH REPORT

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Multi-omics in *MECP2* duplication syndrome patients and carriers

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Abstract

MECP2 duplication syndrome (MDS) is an X-linked neurodevelopmental disorder caused by the gain of dose of at least the genes *MECP2* and *IRAK1* and is characterised by intellectual disability (ID), developmental delay, hypotonia, epilepsy and recurrent infections. It mainly affects males, and females can be affected or asymptomatic carriers. Rett syndrome (RTT) is mainly triggered by loss of function mutations in *MECP2* and is a well described syndrome that presents ID, epilepsy, lack of purposeful hand use and impaired speech, among others. As a result of implementing omics technology, altered biological pathways in human RTT samples have been reported, but such molecular characterisation has not been performed in patients with MDS. We gathered human skin fibroblasts from 17 patients with MDS, 10 *MECP2* duplication carrier mothers and 21 patients with RTT, and performed multi-omics (RNAseq and proteomics) analysis. Here, we provide a thorough description and compare the shared and specific dysregulated biological processes between the cohorts. We also highlight the genes *TMOD2, SRGAP1, COPS2*, *CNPY2*, *IGF2BP1, MOB2, VASP, FZD7, ECSIT and KIF3B* as biomarker and therapeutic target candidates due to their implication in neuronal functions. Defining the RNA and protein profiles has shown that our four cohorts are less alike than expected by their shared phenotypes.

Abbreviations: BH, Benjamini–Hochberg; BP, Biological Process; CEIC, Comitè d'Ètica d'Investigació Clínica; CPM, Counts per million mapped reads; DE, differential expression; DEGs, differentially expressed genes; DEPs, differentially expressed proteins; GO, Gene Ontology; GRCh37/hg19, human reference genome; GSEA, gene set enrichment analysis; ID, intellectual disability; IL-1R, interleukin-1 receptor; IRAK1, Interleukin-1 receptor-associated kinase 1; ISCIII, Instituto de Salud Carlos III; KEGG, Kyoto Encyclopedia of Genes and Genomes; LTP, long-term potentiation; M10, mother number 10; MDS, MECP2 duplication syndrome; MECP2, Methyl-CpG-binding protein 2; NR2A, NMDAR subunit 2A; OMIM, Online Mendelian Inheritance in Man; ORA, Overrepresentation analysis; PKC, protein kinase C; RNAseq, Sequencing of RNA; RP, Reactome pathway database; RTT, Rett syndrome; SI, supplementary information; TLR, Toll-like receptor; WP, WikiPathways; XCI, X chromosome inactivation.

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1 | INTRODUCTION

MECP2 duplication syndrome (MDS; OMIM#300260) is an X-linked neurodevelopmental disorder that mainly affects males. It is a rare syndrome with just over 600 cases reported worldwide (Pascual-Alonso et al., 2021). MDS is characterised by intellectual disability (ID), developmental delay, infantile hypotonia, epilepsy, poor or absent speech, progressive lower extremity spasticity, recurrent infections, gastrointestinal problems, autistic features and mild dysmorphic features (Ta et al., 2022). Females harbouring the *MECP2* duplication present a variable phenotype ranging from asymptomatic carriers to affected girls, depending on the X chromosome inactivation (XCI) status. *MECP2* duplications can be de novo or inherited from carrier mothers who are often asymptomatic, but some cases with neuropsychiatric symptoms or learning difficulties have been reported (Bijlsma et al., 2012).

MDS is caused by the duplication of, at least, the genes *MECP2* (OMIM*300005) and *IRAK1* (OMIM*300283). Duplication locations, sizes, gene contents and dosages are specific to each family (Pascual-Alonso et al., 2021). Unfortunately, no clear genotype–phenotype correlation has been found to date.

Methyl-CpG-binding protein 2 (*MECP2*) is located on the long arm of the X chromosome (Xq28) and undergoes XCI in females. The resulting protein, MeCP2, is ubiquitously expressed, with the MeCP2_e1 isoform being predominant in the brain. *MECP2* is a transcriptional regulator, chromatin remodeller, it interacts with RNA splicing machinery and microRNA processing machinery and participates in neuronal development, maturation and synapse formation (Chahrour et al., 2008; Gulmez Karaca et al., 2019; Sandweiss et al., 2020).

Interleukin-1 receptor-associated kinase 1 (*IRAK1*) is located downstream of *MECP2* and is always duplicated and overexpressed in MDS. *IRAK1* is ubiquitously expressed and is part of the Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) signalling cascades, which are involved in pathogen recognition and the modulation of inflammatory and immune responses (Gottipati et al., 2008), making it a candidate to explain the severe recurrent infections that patients with MDS suffer.

Apart from causing MDS, mutations in *MECP2* trigger Rett syndrome (RTT; OMIM#312750), severe neonatal encephalopathy (OMIM#300673) or X-linked mental retardation syndrome (OMIM#300055). RTT is a neurodevelopmental disorder caused by loss of function mutations in *MECP2*. It is characterised by a normal early development followed by a psychomotor regression that occurs between the first 6 to 18 months of life which includes the appearance of stereotypic hand movements, ID, seizures, breathing disturbances and loss of speech, among others (Neul et al., 2010).

The main affected organ in MDS and RTT is the brain. The impossibility of obtaining human samples from the primarily altered tissue made us focus on a different target tissue. Skin fibroblasts have proven to be a useful resource; the gene expression is less variable than in whole blood and more disease-related genes are expressed (Murdock et al., 2021).

Studying the global transcriptome and proteome is becoming incredibly useful for diagnostics and for research (Stenton et al., 2019). Few transcriptomics studies performed in MDS have been published: five studies in mice models (Ben-Shachar et al., 2009; Chahrour et al., 2008; Chen et al., 2015; Orlic-Milacic et al., 2014; Samaco et al., 2012), one study using modified cell lines (Buist et al., 2022) and one study with two patients with MDS (Sun et al., 2021). No proteomics experiments have been published to date. For RTT, however, there are around 70 published transcriptomic studies and around 30 proteomic studies performed in mice and human samples. Those publications have broadened the knowledge about the biological pathways dysregulated by *MECP2*, but the syndrome's causing pathomechanisms remain unknown.

Here, we present the results of multi-omics (transcriptomics and proteomics) experiments performed in skin fibroblasts of 17 patients with MDS and 10 carrier mothers and compared them with the results of 21 patients with RTT. We aimed to fill the current molecular gap in MDS by delineating the molecular signature of different individuals with the *MECP2* duplication. Knowing which pathways are altered is crucial not only to gain insights into the pathomechanism of the syndrome, but also to find biomarkers and therapeutic targets that could be used in upcoming clinical trials.

2 | MATERIAL AND METHODS

2.1 | Clinical and molecular characterization

Seventeen patients with MDS (15 males and two females), 10 *MECP2* duplication carrier mothers, 21 patients with RTT and 13 healthy controls (six males and seven females) were enrolled in this study; written informed consent was obtained from them all [Table 1; SI_Table 1]. The healthy controls were either mothers of patients who were genetically tested and did not carry their child's mutation, or children with no known disorders or pathologies.

The study was approved by the Sant Joan de Déu Hospital's ethical committee, CEIC (Comitè d'Ètica d'Investigació Clínica): internal code: PIC-219-20. The consent was written and was signed for all caregivers of patients and carriers themselves. Twelve patients with MDS were molecularly characterised in Pascual-Alonso et al. (2020). Six new families were recruited and studied following the same approach. Carrier mothers were also molecularly characterised to confirm the location and size of the duplication. The duplication in carrier mother number 10 (M10) of our cohort was detected in a prenatal test. We considered it to be too early to include her newborn daughter in our cohort since we do not know whether she will develop clinical traits for MDS or not. The 21 patients with RTT have a mutation in *MECP2* and present the necessary criteria for RTT diagnosis (Neul et al., 2010). The clinical severity of patients with RTT was evaluated according to Dr Pineda's clinical severity score (Monrós et al., 2001).

Skin biopsies were obtained from the 61 individuals and primary fibroblast cell lines were established as described in Pascual-Alonso et al. (2023). In short, fibroblast lines were grown on plates with Dulbecco's modified Eagle's medium high glucose with glutamine, supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin, streptomycin and B amphotericin (Thermo Fisher, Waltham, MA, USA). Cultures were kept at 37°C with 5% CO2 in a humidified atmosphere. When 70–80% confluence was reached they were trypsinised, and either resowed on new plates or harvested for subsequent DNA, RNA, or protein extraction. Frozen vials from all the fibroblast lines were entrusted to the Biobanc 'Hospital Infantil Sant Joan de Déu per a la Investigació', which is integrated into the Spanish Biobank Network of ISCIII for the sample and data procurement. DNA was extracted from the fibroblast cell lines using the DNeasy Blood & Tissue Kit, and RNA was extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), both according to manufacturer's instructions. XCI was performed in all female samples as described Allen et al. (1992). XCI was considered skewed with an allele ratio of 80:20 or greater.

2.2 | Transcriptomics

To ensure the quality of the RNAs and discard any undesirable effects due to cell stress, we performed RT-qPCR of five genes of the oxidative respiratory chain (*MT-CO1, MT-CO2, MT-CYB, MT-ND4* and *MT-ATP6)* and two housekeeping genes (*RPLP0* and *ALAS1*), as shown in Pascual-Alonso et al. (2023).

The RNAseq procedure was done with Illumina's TruSeq Stranded mRNA kit for the library preparation (Illumina, California, USA) and it was sequenced on an Illumina NextSeq 500 sequencer (Pascual-Alonso et al., 2023).

Reads were aligned with STAR (v2.4.2a) to the human reference genome (GRCh37/hg19) in a strand-specific manner. The final count matrix was generated by averaging the values of raw counts from different replicates of the same sample. Counts per million mapped reads (CPM) were computed and only genes with at least 1 CPM in more than 50% of samples were kept. The differential expression (DE) analysis was done with DESeq2 (v1.34.0) using the first three principal components for the model construction (Pascual-Alonso et al., 2023). The Benjamini–Hochberg (BH)-corrected p-value of 0.05 was stablished as a threshold to consider differences significant.

2.3 | Proteomics

All proteomics experiments were performed at the BayBioMS core facility at the TUM in Germany, as described by Kopajtich et al. (2021) and analysed as described by Pascual-Alonso et al. (2023). For peptide identification MaxQuant version 1.6.3.4 was used and protein groups were obtained. Missing values were imputed with the minimal value across the dataset. Prior to any analysis, TMT-mass spectrometry (MS) data were adjusted with respect to one identical control sample that was present in each MS batch. Recalibrated intensities were log-transformed for normalisation and proteins that were not detected in all samples were removed. DE analysis was performed using the limma (v3.50.3) package in R considering MS batch as a covariate in the model. A nominal p-value of 0.05 was taken as a threshold to define differentially expressed proteins (DEPs).

TABLE 1 Characterization of the MDS families with fibroblast cell lines. The carrier mothers' duplications coincide with their offsprings' duplications. M10 was included in the carrier's study but her newborn daughter was excluded from the female MDS cohort. XCI was studied in fibroblast DNA or in whole blood when fibroblasts were not available. More detailed information can be found in the SI_Table 1. N.A. stands for "not available". N.I. Stands for "not informative". Y stands for yes and N, for no. the age corresponds to the age at which the skin biopsy was obtained. Some patients were already reported by our group in Pascual-Alonso et al. (2020).

Patient ID	Gender	Age (years)	Epilepsy	Family relations	Duplication origin	Carrier mother's ID	Age (years)	Duplication location	Duplication size (Mb)	Genomic coordinates (GRCh37/hg19)	XCI	Previously reported
P1	M	7	Y	-	Maternal	M1	34	Tandem	0.465	Xq28(153101077-153565901)x2	99:1	Y
P2	M	22	Y	-	Maternal	M2	42	Tandem	0.637	Xq28(152957295-153594098)x2; 1q25.3(183996990-184265525)x3	95:5	Y
P3	M	4	N	-	Maternal	M3	35	Tandem	0.74	Xq28(152832700-153576940)x2	Ni	Y
P4	M	3	N	Cousins	Maternal	M4	32	Tandem	0.416	Xq28(153194797-153611490)x2	76:24	Y
P5	M	1	N		Maternal	M5	34	Tandem	0.416	Xq28(153194797-153611490)x2	70:30	
P6	M	11	Y	-	Maternal	M6	40	Tandem	0.314	Xq28(153287517-153601836)x2	100:0	New
P7	M	4	N	-	Maternal	M7	27	Tandem	0.75	Xq28(153120541-153870079)x2	87:13	New
P8	M	3	Y	-	Maternal	M8	39	Chr Xp	14.3	Xq27.2q28(140928466-155232885)x2	95:5	Y
P9	M	15	N	Brothers	Maternal	M9	39	Tandem	0.511	Xq28(152962751-153473892)x2	Ni	Y
P10	M	12	Y		Maternal	M9	39	Tandem	0.524	Xq28(152949788-153473892)x2	Ni	
P11	M	3	Y	-	Maternal	-		Tandem	0.221	Xq28(153194797-153406233)x2	99:1 (blood)	Y
P12	M	2	N	-	de novo	-		Tandem	0.764	Xq28(153028550-153792888)x2	-	New
P13	M	3	Y	-	Maternal	-		Tandem	0.386	Xq28(153155029-153541192)x2	n.a.	New
P14	M	12	Y	-	de novo	-		Chr 18	2.7	Xq28(152111224-154841455)x2; 15q13.2 (30783615-31089985)x3; 18p11.32 (159550-496915)x1	-	Y
P15	M	3	Y	-	de novo	-		Chr Y	5.8	Xq28(149116213-154929279)x2; Yq11.222q11.23(20826207-28629893)x0	-	Y
P16	F	4	N	-	de novo	-		Tandem	0.346	Xq28(153194797-153541289)x3	51:49	Y
P17	F	5	Y	-	de novo	-		n.a.	0.638	Xq28(153023556-153661653)x3	91:9	New
						M10	41	Tandem	7.94	Xq27.3q28(146407370-154351599)x3	94:6	New

2.4 | Enrichment analysis

Enrichment analysis was performed with clusterProfiler (v4.2.2) and ReactomePA (v1.38.0) R packages. Overrepresentation analysis (ORA) and gene set enrichment analysis (GSEA) were calculated using only significant differentially expressed genes (DEGs) and all expressed genes, respectively. Potentially enriched terms were searched in Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, WikiPathways (WP) and the Reactome pathway database (RP). All genes with CPM greater than 1 in at least 50% of samples and with an existing EntrezID were used as background (11,904 genes for transcriptomics and 5894 genes for proteomics). The cut-off value for considering a significantly enriched term was BH-corrected *p*-value <0.05.

3 | RESULTS

3.1 | Male patients with MDS versus controls

Biased results because of a non-homogeneous distribution of females and males in the cohort were avoided by performing separately differential expression (DE) analysis for males and females with MDS. DE analysis of male patients with MDS against healthy controls showed 2465 differentially expressed genes (DEGs) and 300 differentially expressed proteins (DEPs) (Figure 1a, b; SI_Table 2a,b). From those DEGs and DEPs, 103 genes are significant in both transcriptomics and proteomics analysis (Figure 1c; SI_Table 2c).

Enrichment analysis of transcriptomics and proteomics results revealed several significantly dysregulated biological processes such as the cytoskeleton, neuronal system, vesicular activity, immune system and Wnt and NF-kB signalling cascades (Figure 2, Figure 3; SI_Table 3a,b,c).

DE analysis was also performed stratifying the patients according to their duplications' size (threshold at 1 Mb) and location (in tandem versus outside Xq28) to see whether a correlation with patient severity or prognosis could be found (SI_Table 2d). No specific dysregulated molecular pathways were found in any group.

3.2 | Female patients with MDS versus controls

We analysed the two girls with MDS that we characterised (Table 1). DE analysis of the female patients with MDS against female controls revealed 5720 DEGs and 493 DEPs (SI_Table 2e,f). Two hundred and forty-nine genes are significantly differentially expressed at the mRNA and protein levels (SI_Table 2g). An enrichment analysis with the 249 common genes showed that 26% are related mainly to translation (SI_Table 3f). Translation related DEGs are dysregulated in both directions but

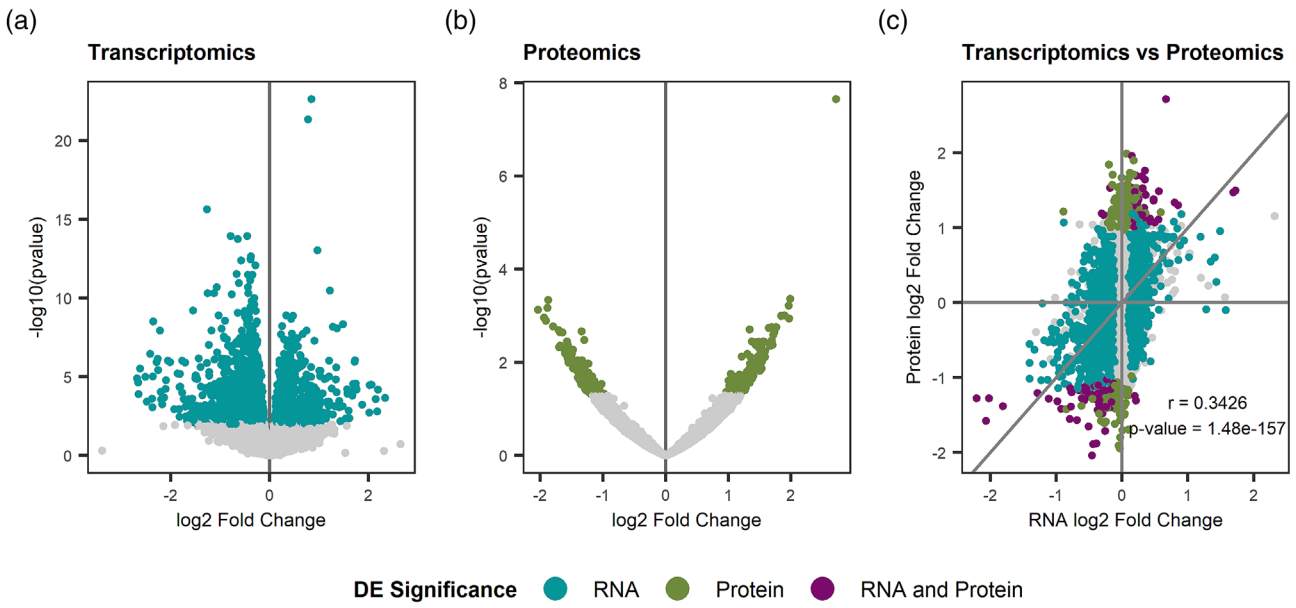

FIGURE 1 Summary of the results of the male patients with MDS versus male healthy controls analysis. (a) RNAseq DE analysis results. The coloured genes are considered differentially expressed, passing a threshold of BH < 0.05. (b) Proteomics DE analysis results. The coloured proteins are considered differentially expressed, passing a threshold of nominal *p*-value <0.05. (c) An integrated view of the transcriptomics and proteomics results. The genes that are significant at both analyses are coloured in purple.

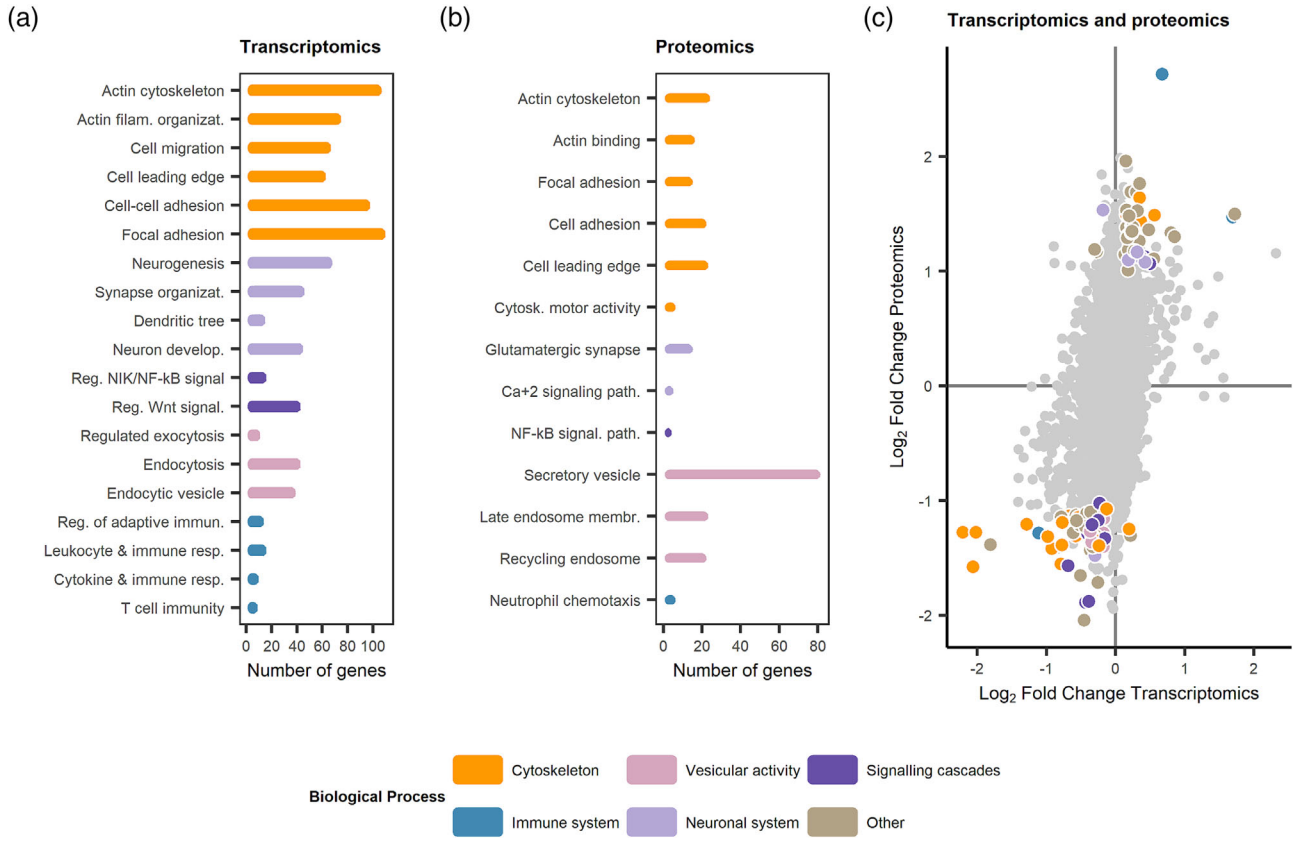

FIGURE 2 Summary of the common findings of the analysis of male patients with MDS versus male healthy controls. (a) Enrichment analysis results for the transcriptomics experiment, coloured by biological process (BP). (b) Enrichment analysis results for the proteomics experiments, coloured by BP. (c) Common significant genes for transcriptomics and proteomics DE analysis results. The common 103 DEGs and DEPs are coloured by BP.

DEPs are predominantly upregulated. In addition, splicing-related terms are enriched with downregulated DEGs but upregulated DEPs.

A comparison between the results obtained for the male and female cohorts with MDS showed that there are only three genes significantly dysregulated in both cohorts and in both omics that are not present in the carrier cohort: *ABCC4*, *STK17B* and *MYO1C*. Similarly, there are some shared biological pathways between male and female cohorts with MDS, such as cellular adhesion, vesicular activity and synapses, all of which are downregulated (SI_Table 3a–f).

3.3 | MECP2 duplication carriers versus controls

The DE analysis of the 10 carrier mothers against female healthy controls showed 2888 DEGs and 635 DEPs (SI_Table 2h,i). In total, 177 genes are dysregulated at the mRNA and protein levels (SI_Table 2j) and enrichment analysis of those common genes showed few terms (SI_Table 3i).

When considering the DEGs and DEPs that are not shared between males with MDS and carrier mothers, terms related to the cytoskeleton were still present for males, while ribosome binding and phagocytic activity-related terms appeared for the carrier mothers. General enrichment analysis of carrier mothers has shown multiple dysregulated genes related to the cell cycle, splicing, nuclear transport, protein folding and degradation and phagocytosis at the mRNA or protein levels, with most of them being downregulated (SI_Table 3g,h).

Our female patients with MDS and carrier mothers share a high percentage of DEGs, but we can see no common enriched terms when considering the genes that are differentially expressed in both omics and are shared between the two cohorts (SI_Table 3d–i).

3.4 | Male MDS versus female patients with RTT

Since *MECP2* is altered in both RTT and MDS, we compared the DE analysis results of the male cohort with MDS and a female cohort with RTT (SI_Table 2k–m,

SI_Table 3k–m). At the transcriptomics level, 721 significant DEGs were shared between the two analyses (SI_Table 2n). At the proteomics level, only 12 DEPs were common (SI_Table 2o) and only two genes were significantly altered in both omics and in both syndromes. We specifically queried the DEGs that are dysregulated in the same or opposite directions (SI_Table 3j). The 82 DEGs that are downregulated in RTT and upregulated in MDS (which corresponds to MeCP2 expression levels in these patients) are significantly enriched with processes involved in mRNA processing, mRNA splicing and DNA replication.

The 100 DEGs upregulated in RTT and downregulated in MDS are related to signalling cascades such as Wnt, BMP and TGFß, cell adhesion, cell projection organisation and cell motility. Enrichment analysis for the commonly upregulated DEGs in both syndromes showed no enriched terms, but the analysis for the downregulated DEGs showed cytoskeleton and some synapse-related terms [SI_Table 3j].

4 | DISCUSSION

4.1 | Male patients with MDS: altered pathways

4.1.1 | Cytoskeletal functions

The most recurrently enriched terms in male patients with MDS are related to actin cytoskeletal functions, specifically to processes involved in cell migration, cell adhesion and filament organisation. From these 103 significant DEGs and DEPs, almost a third are related to the cytoskeleton and most of them are downregulated (Figure 3a). The function of some of those genes occurs at the neuronal level (Table 2); for example, *TMOD2* (OMIM*602928) regulates dendritic arborisation and *SRGAP1* (OMIM*606523) regulates neuronal differentiation; both are upregulated. *MOB2* (OMIM*611969) regulates neurite formation and *VASP* (OMIM*601703) regulates dendritic spine morphology,

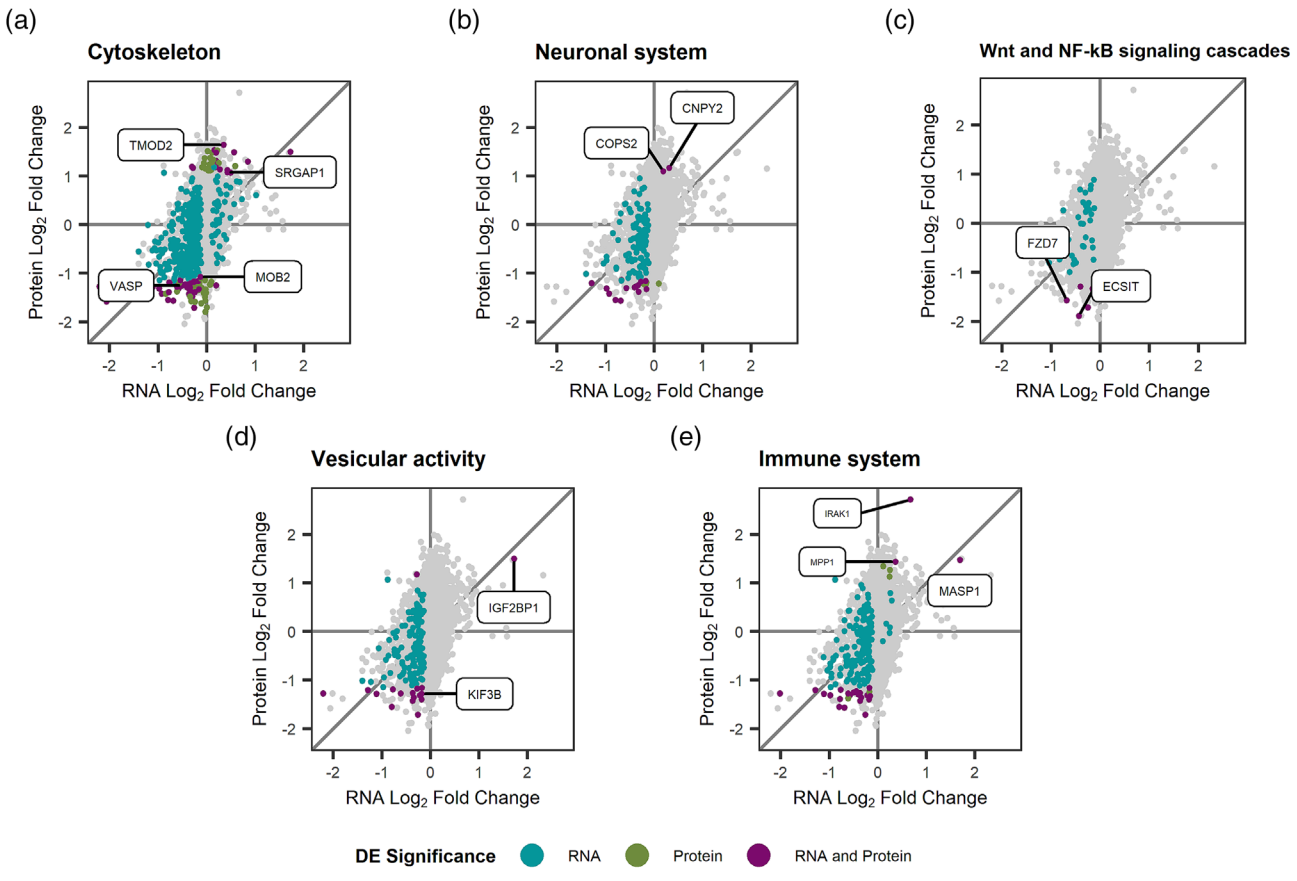

FIGURE 3 Summary of the main biological processes found enriched in the analysis of male patients with MDS versus male healthy control analysis. (a) Significant DEGs and DEPs related to cytoskeleton (coloured in the dotplot). (b) Significant DEGs and DEPs related to the neuronal system. (c) Significant DEGs and DEPs related to Wnt and NF-kB signalling cascades. (d) Significant DEGs and DEPs related to vesicular activity. (e) Significant DEGs and DEPs related to the immune system.

TABLE 2 Interesting significantly DE genes and proteins from the analysis of male patients with MDS against healthy male controls.

Gene	Expression	Biological process
TMOD	Upregulated	Cytoskeleton
SRGAP1	Upregulated	Cytoskeleton
MOB2	Downregulated	Cytoskeleton
VASP	Downregulated	Cytoskeleton
COPS2	Upregulated	Neuronal system
CNPY2	Upregulated	Neuronal system
FZD7	Downregulated	Signalling cascade
ECSIT	Downregulated	Signalling cascade
KIF3B	Downregulated	Vesicular transport
IGF2BP1	Upregulated	Vesicular transport
IRAK1	Upregulated	Immune system
MASP1	Upregulated	Immune system
MPP1	Upregulated	Immune system

axon guidance and neuronal migration, and both are downregulated. It has been thoroughly described that cytoskeletal alterations lead to neuronal malfunction in cognitive and neurodevelopmental disorders such as RTT, autism or Fragile X (Fortin et al., 2012; Liaci et al., 2021; Verpelli & Sala, 2012). The dysregulation of genes and proteins related to actin dynamics and cell adhesion can alter the dendritic spine morphology and synapse plasticity needed for proper synapse function (Betancur et al., 2009; Fortin et al., 2012; Verpelli & Sala, 2012).

4.1.2 | Neuronal system

Even though we have performed our studies in fibroblast tissue, we also identified some terms related directly to the neuronal system in the enrichment analysis, which are mainly downregulated (Figure 3B). Two of the genes that fall into that category are *COPS2* (OMIM*604508), which participates in neuronal differentiation in the early stages, and *CNPY2* (OMIM*605861), which regulates neurite outgrowth; both are upregulated. In MDS mouse models, the dendritic spine density is initially higher but falls below normal levels around the same age as the neurobehavioral traits emerge (Jiang et al., 2013). The genes that we have found to be significantly differentially expressed could be interesting candidates for direct study in neuronal tissues of patients with MDS.

4.1.3 | Wnt and NF-κB signalling cascades

Wnt and NF-κB signalling cascades have also been shown to be enriched with downregulated DEGs and DEPs (Figure 3C). The Wnt signalling cascade takes part in neuronal development, axon and dendrite branching and synapse formation (Armenteros et al., 2018; He et al., 2018), whereas the NF-κB pathway regulates neurogenesis, dendritic complexity, axon guidance and peripheral nerve myelination, among others (Gutierrez & Davies, 2011). NF-kB has been reported to be upregulated in Mecp2-null mice and in Irak1-overexpressing neurons (Kishi et al., 2016). Also, partial silencing of the aberrant NF-kB signalling improves the dendritic phenotype of the RTT mouse model and expands its lifespan. *FZD7* (OMIM*603410), a Wnt protein receptor required for spine plasticity and the migration of glutamatergic neurons, as well as *ECSIT* (OMIM*608388), which regulates the NF-κB pathway, are significantly downregulated in our cohort. These signalling cascades might be impaired and could compromise proper synapse function and axon formation.

4.1.4 | Vesicular transport

Terms related to vesicular transport are also enriched with downregulated DEGs and DEPs (Figure 3D). These genes transport different kinds of cargos. *KIF3B* (OMIM*603754) is downregulated and transports vesicles containing the NMDAR subunit 2A (NR2A), which is required for adequate synaptic plasticity needed for learning and memory (Alsabban et al., 2020). In addition, KIF3B inhibition results in an increased spine density and *Kif3b* haploinsufficient models have an upregulated long-term potentiation (LTP) (Alsabban et al., 2020; Joseph et al., 2020). Both features have been reported in MDS mouse models (Jiang et al., 2013; Na et al., 2012). *IGF2BP1* (OMIM*608288) is upregulated is involved in growth cone migration, dendritic branching and synapse formation in neurons, and also transports transcripts required for axonal regeneration on adult sensory neurons (Núñez et al., 2022). Altogether, our data emphasise a disruption in vesicle formation, cargo uptake and secretion.

4.1.5 | Immune system

Several processes related to the immune system are also significantly enriched with mainly downregulated DEGs and DEPs (Figure 3E). Patients with MDS suffer from recurrent infections and elements of their immune system have been reported to be impaired (Bauer et al.,

2018). Recurrent infections, together with epilepsy, are some of the major factors that trigger regression and cause death in patients with MDS. Among our consistently dysregulated genes and proteins, *IRAK1, MASP1* and *MPP1* are upregulated; the first two are involved in innate immunity function and *MPP1* in neutrophil polarity. It has been hypothesised that the overexpression of *IRAK1* could be key to understanding the malfunction of these children's immune systems. Recently, Gottschalk et al. saw no significant difference in IL-6 and IL-8 production in some patient with MDS samples (Gottschalk et al., 2022).However, the NF-kB pathway by TLR and IL-1R signalling should be further studied in several tissues of patients with MDS to determine whether it is implicated at the neuronal and immunological level in MDS.

4.2 | Male patients with MDS: grouped by duplication size and location

It is thought that part of the phenotypic variability of patients with MDS can be explained by the size, location and content of the duplication, characteristics that are exclusive to each family. No clear genotype–phenotype correlation has been found to date, although it has been reported that larger duplications and triplications tend to lead to a more severe phenotype (Del Gaudio et al., 2006). In our cohort with MDS, we have no triplications and all the duplications in tandem have a size smaller than 1 Mb, whereas the duplications outside the Xq28 region are larger than 1 Mb. No significant enriched terms or dysregulated molecular pathways were found when patients with duplications larger than 1 Mb and outside Xq28 were compared against patients with duplications smaller than 1 Mb and in tandem. Larger cohorts, especially with large and translocated duplications, should be considered for this approach.

4.3 | Female patients with MDS: altered pathways

4.3.1 | Translation

Around a quarter of the shared DEGs and DEPs in female patients with MDS are involved in translation. Buist et al. recently reported an impairment in protein translation and mTOR signalling in RTT human brains but not in a cell model overexpressing *MECP2* isoforms (Buist et al., 2022). Those findings are consistent with our data, since no relevant impairment has been detected in our male patients with MDS, but our females, whose phenotype is milder, present dysregulation of the genes related to translation, which are mainly upregulated. All the DEGs and DEPs related to translation, together with those found in the enriched terms involved in proteasome regulation and nonsense mediated decay, could reflect a cellular compensation, explaining why our females have a milder phenotype and a different molecular signature than our males with MDS. To further confirm the status of protein translation machinery in MDS, other MDS models and tissues should also be tested.

4.3.2 | mRNA processing

Splicing-related terms have downregulated DEGs but upregulated DEPs. The tight regulation of alternative splicing is crucial for neurodevelopment and also for synaptic plasticity (Shah & Richter, 2021). *MECP2* interacts with splicing factors and regulates splicing (Li et al., 2016). However, it has recently been stated that *MECP2* can only regulate the alternative splicing of specific genes rather than doing it in a global way (Chhatbar et al., 2020). Splicing dysregulations have been found in RTT and in other monogenic intellectual disabilities (Shah & Richter, 2021), so the affected transcripts might be implicated in similar biological functions, explaining why those splicing defects are recurrently detected in disorders with overlapping traits.

In any case, a bigger female cohort with MDS and more age-matched controls should be included to improve the reliability of these results and discard any possible interference caused by age differences in this cohort.

4.4 | Comparison between female and male patients with MDS

Only three genes were differentially expressed in male and female patients with MDS in both omics: *ABCC4*, *STK17B* and *MYO1C*. *ABCC4* (OMIM*605250) is needed for dendritic cell migration, which are key initiators of the immune response (Van de Ven et al., 2008). *ABCC4* is downregulated in males with MDS but upregulated in females. Those results are consistent with the immune system dysfunction reported in our males but not in our females with MDS. *STK17B* (OMIM*604727) is a downstream effector of protein kinase C (PKC) in the immune system. PKC is also involved in dendrite development and synapse plasticity in Purkinje cells. Reduced levels of STK17B in mouse models have been shown to protect Purkinje cell dendrites from the negative impact of the activation of PKC signalling. STK17B is upregulated in males with MDS but downregulated in females. Finally,

MYO1C (OMIM*606538), which is downregulated in male and female cohorts with MDS, is a myosin involved in cytoskeletal organisation and vesicle trafficking and when depleted, induces fragmentation of the Golgi complex, the loss of cellular F-actin and a delay in transport (Capmany et al., 2019). *MYO1C* is also involved in the recycling of glucose transporters in response to insulin (Åslund et al., 2021).

Male and female cohorts with MDS share some biological pathways, such as cellular adhesion, vesicular activity and synapses; which are downregulated. These commonly altered biological processes can contribute to the dysregulation in neuronal architecture and synapse function that occurs in patients with MDS, even if it is to a different degree. Therefore, a larger female cohort with MDS should be analysed. On the other hand, no enriched terms related to immune system deficiencies were found in females. These data reflect our children's phenotype, since only our male patients are affected by recurrent infections.

4.5 | *MECP2* duplication carriers: altered pathways

The enrichment of the 177 commonly dysregulated DEGs and DEPs shows few terms. The terms have been classified as cytoskeleton and vesicular organisation-related, but they are annotated based on the Cellular Component GO domain; thus, no specific information about any altered biological processes is observed. These results show that even though multiple DEGs and DEPs exist at the mRNA and protein levels, no evident dysregulation is detected in the carriers from a multi-omics perspective. This could explain the normal and healthy phenotype that our *MECP2* duplication carriers present, although they cannot be considered control individuals for the multiomic study.

4.6 | Comparison between *MECP2* duplication carriers and male patients with MDS

We looked for specific terms that are enriched in *MECP2* duplication carriers and absent in the male patients with MDS, since those could be rescuing the MDS phenotype. At mRNA or protein levels we found differentially expressed genes related to cell cycle, splicing, nuclear transport, protein folding, protein degradation and phagocytosis, which are mainly downregulated and missing in male patients with MDS. (SI_Table 3 a,b,c,g,h,i). The genes that are not present in males with MDS could be implicated in cellular compensation against the effects of the duplication.

In addition, since carriers possess the duplication, it could be possible that some disease relevant genes are changing their expression in the same direction that in the patients but at a level that is not sufficient to reach phenotypic relevance. Thus, studying the genes that significantly move in the same direction in male and female patients and in carriers, as compared with healthy controls, could reveal interesting correlations between expression levels and phenotype severity. A bigger female cohort with MDS would be helpful for this study.

4.7 | Comparison between *MECP2* duplication carriers and female patients with MDS

Despite numerous shared DEGs, no common enriched terms were found between female cohorts at both omic levels. The differences at transcriptomic and proteomic levels between them could explain why some females can compensate the effect of the duplication and remain asymptomatic carriers. The altered DEGs and DEPs linked to RNA transcription, translation, and protein processes might be essential for this. Skewed XCI might lead to fewer cells with the duplication, contributing to the carriers' asymptomatic nature. Yet, DEGs and DEPs in our carriers indicate that XCI alone does not ensure proper molecular regulation. The mechanism causing skewed XCI in some females remains elusive. Analysing a broader female cohort with *MECP2* duplication, categorized by phenotype, is vital to decipher molecular signatures and address these uncertainties.

4.8 | Comparison between male patients with MDS and patients with RTT

The opposing expression levels of *MECP2* between RTT and MDS made it interesting to compare the DE results obtained from both omics studies. However, only two genes, *MYO1C* and *HARS2*, were commonly dysregulated in both syndromes and omics. *MYO1C* is consistently downregulated. *HARS2* (OMIM*600783) is a mitochondrial histidyl-tRNA synthetase 2. At the RNA level, it is upregulated in patients with MDS and downregulated in patients with RTT, whereas it is upregulated in both sets of patients at the protein level. Having only two common genes demonstrates that these patients are not as comparable as thought at the molecular level, at least in fibroblast tissue. The downstream effect of *MECP2* seems to be quite different depending on its dose.

However, it should be noted that some shared DEGs have been detected at the mRNA level which highlight biological processes that could be involved in causing at least some of the features of RTT and MDS.

For example, we have detected 100 DEGs that are upregulated in RTT and downregulated in MDS mRNA, which present enriched terms related to the Wnt, BMP and TGFß signalling cascades. These signalling pathways participate in neurogenesis regulation, myelin synthesis and synapse formation (Armenteros et al., 2018; Fjodorova et al., 2020; He et al., 2018; Pascual-Alonso et al., 2023), functions that, when altered, could explain the malfunction of the neuronal tissues in MDS and RTT. Wnt, BMP and TGFß are also implicated in osteoblast regulation and the maintenance of cartilage (Finnson et al., 2012; Liu et al., 2022; Lowery & Rosen, 2018), and scoliosis and bone fractures have been reported in these syndromes (Pascual-Alonso et al., 2023; Pecorelli et al., 2021; Ta et al., 2022). The dysregulation found in the Wnt, BMP and TGFß signalling pathways could be related to the problems found in the skeletal system of patients with RTT and patients with MDS since childhood. Enrichment analysis in those 100 DEGs also revealed terms related to cell adhesion, cell projection organisation and cell motility. An altered cytoskeleton seems to be implicated in the deficient synaptic activity of patients with MDS and patients with RTT.

Zoghbi's group reported that the majority of the dysregulated genes found in male *MECP2*-Tg and *Mecp2*-null mice hypothalamus and cerebella were shared between both models and that most of them were upregulated (Ben-Shachar et al., 2009; Chahrour et al., 2008). In our cohort, however, less than 30% of the DEGs are common between patients with MDS and patients with RTT and the gene dysregulation occurs evenly in both directions. The integrative transcriptomics analysis of 43 mouse studies performed by Trostle et al. also showed an even distribution of the dysregulated genes (Trostle et al., 2023). The limited correlation of the findings between both species and tissues makes it necessary to confirm the results in patient-derived samples.

The commonly upregulated DEGs in both MDS and RTT showed no enriched terms, indicating that all those DEGs are involved in different biological functions. Enrichment analysis for the consistently downregulated DEGs showed cytoskeleton- and synapse-related terms. The common DEGs that do not follow the expected behaviour for a gene regulated by MeCP2 in these two syndromes could be secondary or indirect effects that cannot be traced with the RNAseq technique.

At the proteome level, only 12 proteins are shared, as described in Pascual-Alonso et al. (Pascual-Alonso et al., 2023). Overall, a limited correlation between transcriptomics and proteomics experiments was found, similar to other groups (Pacheco et al., 2017; Vogel & Marcotte, 2012). This is partly due to the producing and degrading rates of the molecules but also because the characteristics of the experiments, such as the resolution of the techniques, which complicates the generation of more comparable results between these two omics (Vogel & Marcotte, 2012).

5 | CONCLUSIONS

Our male cohort with MDS shows the significant upregulation of the genes *TMOD2, SRGAP1, COPS2*, *CNPY2* and *IGF2BP1* and the significant downregulation of *MOB2, VASP, FZD7, ECSIT* and *KIF3B* at the mRNA and protein levels. They have been published to be involved in neurite formation, dendritic arborisation, synaptic plasticity and neuronal differentiation and migration, thus being implicated in the neuronal dysfunctions reported in MDS. Those genes are expressed in brain tissue and are not associated with any other disorder presenting ID or neurodevelopmental delay, making them candidates for therapeutic targets and diagnostic biomarkers of MDS. In particular, *KIF3B* seems to be a promising candidate, as its inhibition leads to increased spine density and upregulated LTP, two features that have been seen in the brains of MDS mouse models. Therefore, studying these genes in neuronal models derived from patients with MDS would be recommended. In addition, fibroblasts are involved in maintaining epithelial and immune homeostasis in the lung and intestine, and some of the core features of MDS are associated with these organs. Now that we have reported dysregulated genes and pathways in patient-derived fibroblasts, it would be interesting to find out how the genetic changes we have found alter the proper function of cells in these organs. The study of intestinal and lung biopsies may shed some light on this issue.

We have shown distinct transcriptomics and proteomics profiles for males and females with MDS, *MECP2* duplication carriers, and patients with RTT. MDS girls in our study exhibit a mild phenotype, and the limited overlap of DEGs and DEPs between male and female cohorts with MDS highlights the sex-based phenotypic differences in this syndrome. While *MECP2* duplication carriers and females with MDS have a similar mRNA molecular signature, they differ at the protein level. We

believe dysregulated DEGs and DEPs might compensate for duplication effects in carrier mothers, but not entirely in females with MDS. However, our female MDS sample size is limited, which might not fully reflect the syndrome's molecular perturbations. Addressing sample size challenges in rare diseases may involve integrating data from multiple published studies. Multi-omics has been invaluable for understanding syndromes, identifying biomarkers, and pinpointing therapeutic targets.

Definitively, multi-omics has proven to be a useful technique to gain insight into the altered genes and molecular processes of a syndrome, as well as to find biomarkers and therapeutic targets.

AUTHOR CONTRIBUTIONS

Ainhoa Pascual-Alonso: Data curation; formal analysis; investigation; methodology; software; validation; writing—original draft; writing—review and editing. **Clara Xiol:** Data curation; formal analysis; investigation; methodology; software; validation; writing—review and editing. **Dmitrii Smirnov:** Investigation; methodology; software; validation; writing—review and editing. **Robert Kopajtich:** Data curation; formal analysis; methodology; software; validation; writing—review and editing. **Holger Prokisch:** Conceptualization; formal analysis; investigation; methodology; software; validation; visualization; writing—review and editing. **Judith Armstrong:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; resources; supervision; validation; visualization; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

DATA SHARING STATEMENT

Our data is sensitive and protected by the Data Protection Officer (DPO); therefore, we cannot share it in a public repository but rather upon request. Our bioinformatics team at SJD is responsible for safeguarding the data.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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