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Proteomic analysis of the Rett syndrome experimental model *mecp2*^{Q63X} mutant zebrafish

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ABSTRACT

Rett syndrome (RTT) is a severe genetic disorder resulting from mutations in the X-linked *methyl-CpG-binding protein 2* (*MECP2*) gene. Recently, a zebrafish carrying a *mecp2*-null mutation has been developed with the resulting phenotypes exhibiting defective sensory and thigmotactic responses, and abnormal motor behavior reminiscent of the human disease. Here, we performed a proteomic analysis to examine protein expression changes in *mecp2*-null *vs.* wild-type larvae and adult zebrafish. We found a total of 20 proteins differentially expressed between wild-type and mutant zebrafish, suggesting skeletal and cardiac muscle functional defects, a stunted glycolysis and depleted energy availability. This molecular evidence is directly linked to the *mecp2*-null zebrafish observed phenotype. In addition, we identified changes in expression of proteins critical for a proper redox balance, suggesting an enhanced oxidative stress, a phenomenon also documented in human patients and RTT murine models. The molecular alterations observed in the *mecp2*-null zebrafish expand our knowledge on the molecular cascade of events that lead to the RTT phenotype.

Biological significance: We performed a proteomic study of a non-mammalian vertebrate model (zebrafish, *Danio rerio*) for Rett syndrome (RTT) at larval and adult stages of development. Our results reveal major protein expression changes pointing out to defects in energy metabolism, redox status imbalance, and muscle function, both skeletal and cardiac. Our molecular analysis grants the *mecp2*-null zebrafish as a valuable RTT model, triggering new research approaches for a better understanding of the RTT pathogenesis and phenotype expression. This non-mammalian vertebrate model of RTT strongly suggests a broad impact of Mecp2 dysfunction.

1. Introduction

Rett syndrome (RTT, MIM 312750), a progressive neurodevelopmental disorder with a frequency of approximately 1:10,000 live births, is a leading cause of severe intellectual disability in the female [1]. The typical disease shows a period of 6 to 18 months of an apparent normal neurodevelopment followed by regression and progressive loss of acquired cognitive, social and motor skills [2]. *De novo* mutations, altering the function of the X-linked methyl-CpG binding protein 2 (*MECP2*) gene, are the main cause of RTT [3]. Although the *MECP2* functions are yet to be fully clarified, the encoded protein can act as either a transcriptional repressor or activator by binding to methylated DNA [4], and is able to modulate alternative RNA splicing [5] and miRNA processing [6].

Several experimental mammalian models of RTT recapitulating several features of the disease have been developed [7]. Non-mammalian models of RTT have been recently described, including *Drosophila* and *Danio rerio* [8,9]. In particular, zebrafish has recently gained much attention as a vertebrate model for human neurodevelopmental and neurodegenerative diseases [10], showing a number of unique advantages that include its strong genetics (*i.e.*, amenable to molecular manipulations of its genome) [11,12], a large repertoire of well-studied behaviors [13], imaging capabilities in larvae and access to early nervous system

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development [14]. The *mecp2*-null zebrafish model mirrors the defective motor behavior and sensory response observed in RTT patients [9,15].

Most studies have focused on defects linked to the development and maintenance of brain networks [16]. However, the fact that *MECP2* is broadly expressed, from the early stages of development with expression levels increasing progressively and becoming particularly enriched in neural tissues [17], and the large set of symptoms described in human patients [18], argues for pleiotropic roles of *MECP2*.

Hence, by using a proteomic approach in whole tissue samples from larvae and adults, we demonstrate that *mecp2*-null zebrafish exhibits changes in the expression of proteins mainly linked to the balance of the redox status, energy metabolism and muscle function.

2. Materials and methods

2.1. Animals

Zebrafish larvae and adults were maintained at 28.5 °C on a 14–10 h on/off light cycles. Larvae were grown accordingly to Westerfield [19]. All experimental procedures were performed at room temperature (21–23 °C). To minimize the effect of the genetic background variability on our analysis, we used the progeny of wild-type and homozygote mutant issued from incrossed zebrafish heterozygotes for the *mecp2*^{Q63X} null mutation (C to T transition in position 184 of the zebrafish cDNA, leading to the generation of a stop codon). The C184T mutation was identified by PCR using DNA extracted from fin clip (the primers 5′-AAAGGAAAGGCATGATGTGG-3′ and 5′-GTATCGCCAACCTTTTGGAA-3′ flank the position of the mutation), followed by sequencing. The loss of *mecp2* has been previously confirmed by Western blot analysis [15]. Gene sequencing for mutant, heterozygotes and wild type animals was added as Supplementary material 1. The whole tissue samples of WT and *mecp2*^{Q63X} (referred as RTT further in the text), larvae and adults were used for proteomic analysis. All procedures were carried out in compliance with the guidelines of *Le Comité d'Éthique pour l'Expérimentation Animale Charles Darwin*.

2.2. Sample preparation

Adult fishes were homogenized (whole tissues) in 7 M urea, 2 M thiourea and 4% CHAPS, on ice. Four volumes of ice-cold buffer per weight of tissue for the homogenization procedure were added. Approximately 1/4 of complete protease inhibitor cocktail tablet (Roche, ID: 11836153001) per fish was added to the homogenates, subsequently incubated 2 h under agitation at 4 °C. The samples were then centrifuged at ~21,000 ×*g* for 15 min at 4 °C and soluble fractions reserved, snap-frozen and kept at −80 °C until use. Larvae were harvested at 8 dpf and snap-frozen and kept at −80 °C until use. To extract proteins, they were first subjected to 3 freeze-and-thaw cycles of 20 s each. Samples were then ground with a pestle in 1.5 mL tubes and vortexed for 30 s in a lysis buffer consisting of 9 parts 20 mM Tris-HCl, pH 8.5, 20 mM NaCl and 1 part protease inhibitor cocktail. The samples were placed on a shaking table for 20 min at room temperature before spinning down cellular debris at 16100 ×*g* for 10 min at 4 °C. The supernatant was transferred to a fresh tube and frozen in −80 °C until use.

2.3. Two dimension gel electrophoresis (2-DE)

2-DE was performed according to Görg et al. [20]. Briefly, samples (60 μg) were combined with solubilizing buffer containing 8 M urea, 2% w/v CHAPS, 0.3% DTT, 2% immobilized pH gradient IPG buffer (GE Healthcare), and were separated using 18 cm pH 3–10 non-linear IPG strips (GE Healthcare), in an Ettan IPGphor apparatus system (GE Healthcare). After focusing, strips were combined with equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, and 1% w/v DTT for 15 min, followed by equilibrating in equilibration buffer with 4% w/v iodoacetamide and a trace of bromophenol blue for 10 min. The second dimension was performed embedding IPG strips and a molecular weight standard at the top of a 1.5 mm thick vertical polyacrylamide gradient gel (8–16% T) using 0.5% w/v agarose, in an EttanDalt Six Electrophoresis system (GE Healthcare). Protein spots from the 2-DE gels were visualized by silver staining, optimizing the exposure time in order to avoid overexposure of some gels with respect to others. Each sample was carried out in triplicate under the same conditions.

2.4. LC–ESI–MS/MS (proteomic analysis)

After MS compatible silver staining [21], protein spots were carefully excised and subjected to in-gel trypsin digestion according to Shevchenko et al. [22]. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to MS analysis, the peptide mixtures were redissolved in 10 μL of 5% formic acid. Samples were analyzed using a split-free nano-flow LC system (EASY-nLC II, Proxeon, Odense, Denmark) coupled to a 3D-ion trap (model amaZon ETD, Bruker Daltonik, Germany) equipped with an online nano-ESI sprayer (the spray capillary was a fused silica capillary, 0.090 mm o.d., 0.020 mm i.d.). All experiments and the mass spectrometer acquisition parameters were according to Timperio et al. [23]. Acquired collision-induced dissociation spectra were processed in DataAnalysis 4.0, and deconvoluted spectra were further analyzed with BioTools 3.2 software and submitted to Mascot search program (in-house version 2.2, Matrix Science, London, UK). A list of identified proteins (Supplementary Table 1), and the inclusion criteria for protein identification and selection (*i.e.* peptide sequences and the relative score, peptide charge status) are reported (Supplementary Table 2). Regarding validation method for MS/MS searches having a small number of spectra but high MASCOT scores, manual interpretation of peptide fragmentation spectra was used to validate protein assignments.

2.5. Data analysis

Electrophoretic gels were digitized and analyzed by ImageMaster 2D Platinum v7.0 software (GE Healthcare). Spot volume was expressed as a ratio of the percentage volume (%V) detected from the entire gel to minimize differences between samples (normalization). Only spots appearing in all gels of the same group were matched with those of the reference gel. The background was subtracted from all gels using the average-on-boundary method. Results were expressed as mean ± standard deviation to the mean (mean ± SD), and analysis of protein variations was performed by multiple *t*-test, with a False Discovery Rate (*q*) of 0.05 (GraphPad Prism 6.01, GraphPad Software, Inc., CA, USA; MedCalc 12.1.4, MedCalc Software, Mariakerke, Belgium). A two-tailed *p*-value of <0.05 was considered as statistically significant.

3. Results

We found a total of 322 ± 10 spots in wild-type and RTT larvae, and 377 ± 17 spots in wild-type and RTT adults. A cluster of 32 spots, corresponding to 20 proteins, were differentially expressed in homozygote *mecp2*^{Q63X} mutant zebrafish (Table 1, Fig. 1 and Supplementary data). A detailed list of RTT/WT ratios is also reported (Supplementary material 2 and 3).

3.1. Protein expression changes in RTT zebrafish at the larval stage

Significant expression changes at the larval stage were present for 16 proteins, the overwhelming majority of them being decreased as compared to the wild-type counterpart. The two proteins prdx2 and dj1, known to play an important role in *anti*-oxidative stress (OS) processes [24,25], were overexpressed in the RTT zebrafish larvae, whereas apoa1b was underexpressed.

Table 1
Zebrafish proteome: protein pattern changes as a function of Rett disease and stage development.

Protein/spot info.			Comparison	*p*-Value	
Protein	Short name	Spot n.	RTT^L/WT^L ratios	RTT^A/WT^A ratios	RTT^A *vs.* RTT^L
Peroxiredoxin-2	prdx2	1	↑(2.51 ± 0.08)**	↑(1.45 ± 0.06)**	<**0.01****
Protein deglycase DJ-1	dj1	2	↑(1.97 ± 0.07)**	↑(1.87 ± 0.08)**	0.349
Serum transferrin, precursor	tfa	3	n.d.	↓(0.46 ± 0.09)*	**0.013***
Apolipoprotein A-1b, precursor	apoa1b	4	↓(0.39 ± 0.04)*	↓(0.61 ± 0.05)**	0.054
Apolipoprotein A-1b, precursor	apoa1b	5	↓(0.27 ± 0.03)**	↓(0.73 ± 0.13)**	0.055
Beta enolase	eno3	6	↓(0.15 ± 0.02)**	↓(0.52 ± 0.01)	<**0.01****
Beta enolase	eno3	7	↓(0.42 ± 0.03)**	↓(0.58 ± 0.05)*	**0.042***
Beta enolase	eno3	8	↓(0.19 ± 0.02)**	↓(0.33 ± 0.03)**	**0.045***
Aldolase A	aldoa	9	↓(0.44 ± 0.05)*	↓(0.48 ± 0.02)*	0.262
Glyceraldehyde-3-phosphate dehydrogenase	gapdh	10	↓(0.25 ± 0.02)**	↓(0.33 ± 0.06)*	0.089
Glyceraldehyde-3-phosphate dehydrogenase	gapdh	11	↓(0.42 ± 0.05)**	↓(0.38 ± 0.02)**	0.311
Triosephosphate isomerase B	tpi1b	12	↓(0.64 ± 0.02)*	=(0.91 ± 0.13)	0.091
Triosephosphate isomerase B	tpi1b	13	↓(0.40 ± 0.09)*	=(0.69 ± 0.11)	0.128
Triosephosphate isomerase B	tpi1b	14	↓(0.33 ± 0.05)**	↓(0.34 ± 0.06)**	0.972
Malate dehydrogenase, mitochondrial	mdh2	15	↑(1.34 ± 0.01)**	↑(1.24 ± 0.09)*	0.217
Nucleoside diphosphate kinase B	nkz2	16	=(1.03 ± 0.04)	↓(0.44 ± 0.04)**	<**0.01****
Nucleoside diphosphate kinase B	nkz2	17	↓(0.62 ± 0.02)**	↓(0.47 ± 0.02)**	**0.021***
Adenylate kinase 1	ak1	18	=(1.06 ± 0.07)	↓(0.54 ± 0.03)**	<**0.01****
Adenylate kinase 1	ak1	19	disappearance**	↓(0.75 ± 0.02)*	<**0.01****
Myosin heavy 2	myhz2	20	↓(0.47 ± 0.04)*	↓(0.65 ± 0.04)*	<**0.01****
Myosin heavy 4	myhc4	21	↓(0.27 ± 0.03)**	↓(0.52 ± 0.10)**	0.056
Myosin heavy 4	myhc4	22	↓(0.60 ± 0.07)**	↓(0.47 ± 0.02)**	0.051
Myosin heavy 4	myhc4	23	↓(0.41 ± 0.09)**	↓(0.47 ± 0.01)**	0.509
Myosin heavy 4	myhc4	24	↓(0.46 ± 0.03)**	↓(0.49 ± 0.01)**	0.283
Myosin light 1/3	myl1	25	=(1.05 ± 0.07)	↓(0.61 ± 0.02)**	<**0.01****
Tropomyosin alpha-1	tpma	26	=(0.94 ± 0.10)	↓(0.40 ± 0.04)*	**0.027***
Actin alpha-1	acta1	27	↓(0.81 ± 0.05)**	↓(0.22 ± 0.02)**	<**0.01****
Muscle-specific creatine kinase	ckma	28	↓(0.29 ± 0.03)**	↓(0.30 ± 0.05)**	0.961
Troponin I type 2b	tnni2b	29	↓(0.62 ± 0.12)	↓(0.27 ± 0.02)**	0.065
Troponin I type 2b	tnni2b	30	↓(0.76 ± 0.10)	↓(0.48 ± 0.02)**	0.058
Alpha-cardiac actin	actc1a	31	↓(0.25 ± 0.04)**	↓(0.27 ± 0.03)**	0.737
Crystallin beta A2a	cryba2	32	↓(0.56 ± 0.08)**	↓(0.53 ± 0.04)**	0.706

(Table 1 legend) n.d., not detected spot, (↓) decrease, (↑) increase and (=) non-detectable changes. Two-tailed *p*-values <0.05(*) and <0.01(**) are shown. Significant *p*-values are highlighted in bold. Mean ratios (±SD) of spot values (%V) from Rett zebrafish proteome normalized *vs.* mean values of spots from wild type zebrafish proteome are reported in brackets.

Expression of 4 proteins related to glycolysis (eno3, aldoa, gapdh and tpi1b), a gluconeogenic enzyme (mdh2), and 2 adenosine triphosphate (ATP)-related proteins (nkz2 and ak1) were altered. Specifically, eno3, aldoa, gapdh, tpi1b and nkz2 (spot 17) were underexpressed, while expression of mdh2 and ak1 (spot 19) were increased or no longer detectable, respectively.

We observed a decreased expression of proteins related to structural and functional features of the skeletal muscle (myhz2, myhc4, acta1 and ckma). Likewise, actc1a was underexpressed, as well as cryba2. No significant changes were detected for nkz2 (spot 16), ak1 (spot 18), myl1, tpma and tnni2b (see the RTT^L/WT^L ratios, Table 1).

3.2. Differentially expressed proteins in the adult RTT zebrafish

Early changes observed at the larval stage were essentially maintained in the adult RTT zebrafish (Table 1). On the other hand, a few significant variations were evidenced between early and late disease. Specifically, prdx2 was overexpressed, although at a lower extent than in RTT larvae, whereas tfa was underexpressed, although detectable in the adult stage only.

Among glycolysis-related proteins, eno3 is the only protein found to be significantly underexpressed at a lower degree than in larvae (spots 7–8).

Protein spots corresponding to nkz2 and ak1 were generally underexpressed. A decreased expression for almost all the structural and functional muscle proteins was detectable in the adult RTT zebrafish. Specifically, myl1, tpma and actc1a were underexpressed at a higher extent than in larvae, while myhz2 was underexpressed at a lower extent. No significant changes for eno3 (spot 6) and tpi1b (spots 12 and 13) were detected.

3.3. Developmental protein patterns

Similarities between wild-type and RTT zebrafish, in terms of protein expression, were observed for dj1, a total of three glycolysis-related proteins including aldoa, gapdh and tpi1b (spots 9–14), the gluconeogenic enzyme mdh2 (spot 15), skeletal muscle proteins myhc4 and ckma (spots 23, 24 and 28), actc1a (spot 31) and cryba2 (spot 32). In addition, significant differences between wild-type and RTT zebrafish were observed for prdx2 and tfa (spots 1 and 3), glycolysis-related protein eno3 (spots 6–8), ATP-related proteins nkz2 and ak1 (spots 16–19), and skeletal muscle proteins myhz2, myl1, tpma and acta1 (spots 20, 25–27). Non-significant trends for apoa1b, myhc4 (spots 21 and 22) and tnni2b were evidenced.

4. Discussion

Our proteome analysis, at larval and adult stages of the *mecp2*-null zebrafish development, points out on possible pathophysiological explanations behind the observed abnormalities in motor behavior. They include energy depletion, redox imbalance, structural and/or functional muscle deficits, together with a possible vision impairment. The great majority of these biochemical changes occurs at an early stage (*i.e.*, larvae) of the natural history of the disease in the zebrafish. While RTT patients show a major motor dysfunction, the *mecp2*-deficient zebrafish shows a decreased locomotor activity at the larval stage, possibly explained by a reduction of interest in environment exploration as observed hyper-responsive autistic patients [9,26].

Energy and metabolism dysfunction in RTT has been reported both in patients and experimental mouse models [27,28]. Our data reveal an underexpression for specific cytosolic enzymes involved in glycolysis

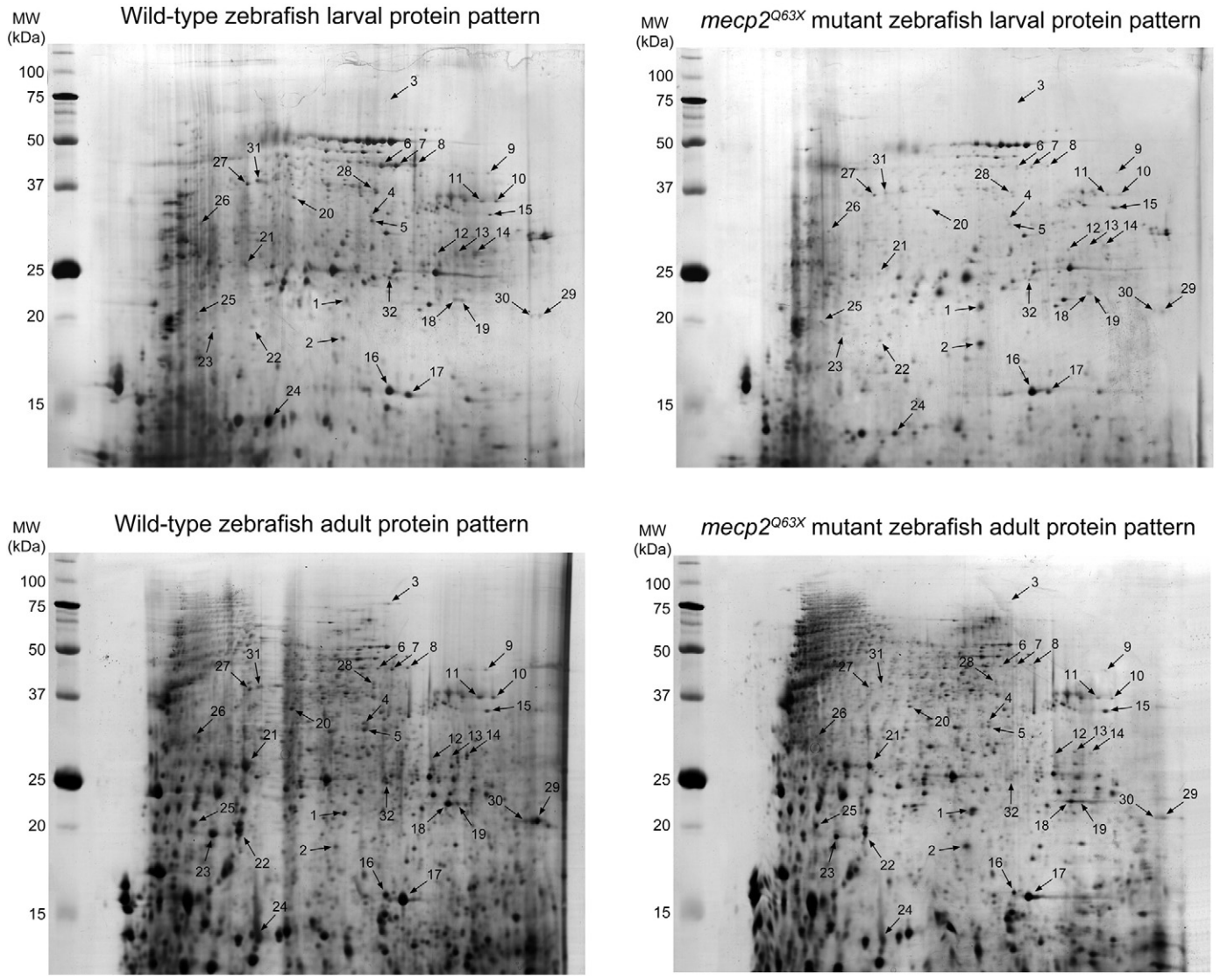

Fig. 1. Zebrafish protein patterns as a function of Rett disease and stage development. Zebrafish proteins were evaluated in terms of expression in the *mecp2*^{Q63X} mutant zebrafish at larval and adult stage and compared with corresponding wild-type larvae and adults. Numbers denoting identified proteins by MS are listed in Table 1. Molecular weight (MW, kDa) is indicated.

and ATP metabolism, thus suggesting a stunted glycolysis and a consequent depleted energy availability. Glycolysis is one of the most important sources of energy in erythrocytes and some types of skeletal muscle fibers [29]. Here, we found a decreased expression of gapdh, a key enzyme in glycolysis, is in line with our prior observation of a decreased protein expression in human RTT erythrocytes [30]. Indeed, multiple glycolytic pathway alterations seem to exist in the model given that eno3, aldoa and tpi1b are significantly underexpressed.

We also evidenced a decreased expression in nkz2 and ak1, two enzymes involved in the ATP metabolism [31]. In particular, reduced brain ATP levels have been reported in *Mecp2*-null mice [32]. Interestingly, an altered glycolysis and mitochondrial respiration is the biological basis for a zebrafish model of Dravet syndrome, a severe genetic form of epilepsy [33]. Notably, epilepsy and electroencephalogram alterations are main feature in human RTT [34]. Therefore, an alteration in either glycolysis and/or ATP metabolism could contribute to the genesis of seizures in RTT. An energy deficit may reflect into the cognitive impairment underlying the observed decrease in thigmotaxis exhibited by the *mecp2* zebrafish model [9]. While our findings trigger future functional measurement of glycolysis in the *mecp2* zebrafish model, it remains unclear whether glycolysis alterations may lead to cognitive impairment in RTT. In a recently developed *Mecp2*-deficient mice hypo-activity and exercise fatigue are suggested as specific peripheral RTT phenotype [35], thus reinforcing our indirect findings of the depleted energy in the currently analyzed RTT zebrafish model. Moreover, a deficient energy metabolism is reported to be associated with neurological manifestations, cognitive impairment and eye abnormalities [36].

In the present study, a surprising decrease in the expression of cryba2, a major structural component of the vertebrate lens, was also evidenced. Whether this finding would play any role in the *mecp2*-deficient zebrafish phenotype, it remains to be clarified. Beta-crystallins are known to be critical for lens clarity and refraction [37]. Likewise, mutations in beta-crystallin genes can lead to nonspecific aggregation of crystallins and cataract formation [38]. Notably, a decreased visual spatial acuity in RTT is reported, together with decreased visual evoked potentials amplitude, especially at late stages of the disorder [39]. Furthermore, our preliminary findings in RTT patients indicate a very high frequency of refractive defects (*i.e.*, astigmatism), and eye misalignment [40]. Taken together, our observations stimulate an in-depth study on lens and anterior eye chamber development, as well as a careful assessment of the visual function in this *mecp2*-null zebrafish model.

An abnormal carbohydrate metabolism in cerebrospinal fluid and blood in RTT patients has been previously reported, relating the increase in lactate, a key gluconeogenic precursor, to breathing abnormalities [41]. In a prior study, about half of RTT patients were reported to exhibit elevated levels of circulatory lactic or pyruvic acid, potentially caused by defects in the efficiency of the respiratory chain and urea cycle complexes, both of which are mitochondrial [42]. Here, the mitochondrial enzyme mdh2 was overexpressed in both larval and adult *mecp2*^{Q63X} mutant zebrafish, thus suggesting the tendency towards alternative energy sources through the gluconeogenic pathway. The close overlap between RTT symptoms and mitochondrial disorders is in line with reports of structural abnormalities and defects in the electron transport chain in mitochondria from skin and muscle biopsies from RTT patients [42].

Biochemical cues for the abnormal redox status inferred in our zebrafish model are represented by markedly increased levels of prdx2 and dj1, two proteins playing an important role in the cell protection against OS [24,25]. Therefore, the observed proteomic changes can be interpreted as a homeostatic response to a status of enhanced OS.

Besides other known functions [43], tfa and apoa1b are fundamental for the maintenance of redox balance homeostasis [44,45]. A lower expression of the serum transferrin has been strongly associated with an increased lipid peroxidation in autism [44], and a down-regulation of the apolipoprotein A1 gene has been previously related to redox imbalance and neurological disorders [45].

Overall, the present findings underscore the critical role of OS in RTT [18,46,47], translatable to the zebrafish *mecp2*-deficient model, being mitochondrial dysfunction a possible main source of aberrant redox homoeostasis [48,49].

Early motor disturbances and skeletal muscle mitochondrial dysfunction are reported in RTT patients [50,51]. Previous findings in a RTT mouse model suggest that mitochondrial abnormalities in the skeletal muscle may contribute to the progressive deterioration in mobility through accumulation of free radicals [49]. Moreover, MeCP2 deficiency is reported to affect skeletal muscle growth and morphology, leading to a pathology picture featuring a disorganized architecture, with hypotrophic fibers and tissue fibrosis in the *Mecp2*-null mice [51]. The *mecp2*^{Q63X} mutant zebrafish shows a decreased expression of key motor protein components, including myosins (myhz2, myhc4 and myl1), and other muscle specific proteins (tpma, acta1, ckma and tnni2b). All these proteins are mechanochemical enzymes containing binding sites for ATP (myhz2 and myhc4), Ca^{2+} (myl1 and tnni2b), acta1 (tpma) and generating ATP (ckma) [52]. Therefore, the combination of a likely decreased ATP availability with multiple skeletal muscle protein deficits could underlie the impaired locomotion activity of the zebrafish RTT model [9].

Of interest, a protein related to cardiac muscle function (actc1a) was underexpressed in the RTT zebrafish, a finding that seems to mirror the subclinical biventricular dysfunction already documented in RTT patients where it shows features resembling the stage B of heart failure [53]. This observation may also suggest a potential contributory role on the significantly increased sudden death rate occurring in the natural history of the human disease [54].

Our study, by demonstrating in the *mecp2*-deficient zebrafish the deregulation of several proteins, illustrates the broad impact of MeCP2 dysfunction, not confined to the central nervous system, and confirms the need of a multi-disciplinary effort to develop new research approaches to further decipher disease pathogenesis and phenotype expression.

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Conflict of interest

The authors declare no conflict of interest.

Transparency document

The transparency document associated with this article can be found, in online version.

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