



## **Multi-omics comparisons of different forms of centronuclear myopathies and the effects of several therapeutic strategies**

Sarah Djeddi, David Reiss, Alexia Menuet, Sébastien Freismuth, Juliana de Carvalho Neves, Sarah Djerroud, Xènia Massana Munoz, Anne-Sophie Sosson, Christine Kretz, Wolfgang Raffelsberger, et al.

### **► To cite this version:**

Sarah Djeddi, David Reiss, Alexia Menuet, Sébastien Freismuth, Juliana de Carvalho Neves, et al.. Multi-omics comparisons of different forms of centronuclear myopathies and the effects of several therapeutic strategies. *Molecular Therapy*, 2021, <10.1016/j.ymthe.2021.04.033>. <hal-03245652>

**HAL Id: hal-03245652**

**<https://hal.science/hal-03245652v1>**

Submitted on 13 Oct 2022

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1   **Multi-omics comparisons of different forms of centronuclear myopathies and the effects of several**  
2   **therapeutic strategies**

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## Abstract

Omics analyses are powerful methods to obtain an integrated view of complex biological processes, disease progression or therapy efficiency. However, few studies have compared different disease forms and different therapy strategies to define the common molecular signatures representing the most significant implicated pathways. Here, we used RNA sequencing and mass spectrometry data to profile the transcriptomes and proteomes of mouse models for three forms of centronuclear myopathies (CNM), untreated or treated with either a drug (tamoxifen), antisense oligonucleotides reducing the level of dynamin 2 (DNM2), or following modulation of dynamin 2 or amphiphysin 2 (BIN1) through genetic crosses. Unsupervised analysis and differential gene and protein expression were performed to retrieve CNM molecular signatures. Longitudinal studies before, at and after disease onset highlighted potential disease causes and consequences. Main pathways in the common CNM disease signature include muscle contraction, regeneration and inflammation. The common therapy signature revealed novel potential therapeutic targets including the calcium regulator sarcolipin. We identified several novel biomarkers validated in muscle and/or plasma through RNA quantification, western blotting and ELISA assays, including ANXA2 and IGFBP2. This study validates the concept of using multi-omics approaches to identify molecular signatures common to different disease forms and therapeutic strategies.

**Keywords:** genetic disease, congenital myopathy, centronuclear myopathy, myotubular myopathy, XLMTM, MTM1, myotubularin, omics, biomarker, myostatin, RNA interference

## Background

50 In recent years, omics strategies (e.g. transcriptome, proteome etc.) have become powerful methods to  
51 obtain an unbiased and integrated view of complex biological processes, disease progression or therapy  
52 efficiency<sup>1,2</sup>. Most previous studies focused on a single disease or therapeutic approach. However, omics  
53 have the potential to identify molecular signatures common to different disease forms or to several  
54 therapeutic strategies. Here, we performed omics analyses in mouse models faithfully mimicking  
55 different forms of centronuclear and myotubular myopathies treated or not with different therapeutic  
56 strategies to identify common disease and therapy signatures.

57 Centronuclear and myotubular myopathies (CNM) are a sub-group of congenital myopathies whose  
58 clinical signs develop around birth. They are rare genetic diseases appearing around birth and with a  
59 strong medical impact on patient survival and quality of life<sup>3,4</sup>. CNM are characterized by generalized  
60 muscle weakness and hypotonia impairing breathing capacity<sup>5</sup>. Histological hallmarks of patients'  
61 muscle include the presence of internal or central nuclei that are normally at the fiber periphery,  
62 aggregation of oxidative staining, hypotrophy of myofibers that also present with a rounder shape,  
63 predominance of oxidative type I fiber, and structural disorganization of sarcomeres and triads<sup>6</sup>. Several  
64 genes were found mutated in different forms of CNM. The most common and severe form, X-linked  
65 CNM which is also called myotubular myopathy (MIM#310400), is due to loss-of-function mutations in  
66 *MTM1* coding for the lipid phosphatase myotubularin<sup>7</sup>. Dominant forms are linked to mutations in  
67 *DNM2* (MIM#160150) coding the large GTPase dynamin 2 implicated in membrane trafficking and  
68 fission<sup>8</sup>. Some *DNM2* mutations lead to a neonatal form, such as the Ser619Leu missense mutation,  
69 while others are associated with later onset<sup>9,10</sup>. Recessive and dominant forms are also due to mutations  
70 in *BINI* (MIM#255200), coding for the membrane curvature remodeling protein amphiphysin 2<sup>11,12</sup>.  
71 These three forms represent the main forms of CNM. Nevertheless, additional genes are implicated in  
72 phenotypes overlapping with CNM such as *RYR1*, *TTN*, *SPEG*, *CACNA1S* or *PYROXD1*<sup>3,13,14</sup>. *SPEG* is  
73 linked to CNM with cardiomyopathy, while the histopathology associated with the other genes combines  
74 internal nuclei with additional defects such as cores or protein inclusions.



75 Previous studies in cellular and animal models and in patients' muscle biopsies for the canonical CNM  
76 forms suggested several pathomechanisms in skeletal muscle, including defects in triad structure and  
77 deficient excitation-contraction coupling, altered organelle positioning and function, abnormal  
78 neuromuscular junction (NMJ), deficient satellite cells, and dysregulation of autophagy<sup>4, 15-17</sup>.  
79 Defects in the genes implicated in the three canonical CNM forms were modelled *in vivo* in different  
80 organisms, ranging from yeast, *C. elegans*, drosophila, zebrafish and mouse<sup>17, 18</sup>. In addition,  
81 spontaneous mutations in either *MTM1* or *BIN1* were found in dogs developing CNM<sup>19-22</sup>. In mice, the  
82 *Mtm1*<sup>-/-</sup> knockout mouse develops a progressive myopathy with a histopathology mimicking patient  
83 hallmarks<sup>23</sup>. Additional *Mtm1* knockout lines were generated and showed a similar phenotype, while the  
84 *Mtm1*<sup>RC/y</sup> knock-in led to a milder phenotype<sup>24-26</sup>. Concerning *Dnm2*, knock-in mice for the most  
85 common mutations in the mildest form (Arg465Trp ; *Dnm2*<sup>RW/+</sup>) or the severe neonatal form  
86 (Ser619Leu ; *Dnm2*<sup>SL/+</sup>) were generated and reproduce a mild or severe muscle weakness, respectively,  
87 with CNM-like histopathology without centralized nuclei<sup>27, 28</sup>. For *Bin1*, full loss of BIN1 in *Bin1*<sup>-/-</sup> mice  
88 is perinatally lethal, preventing the comparison with the other CNM models<sup>29, 30</sup>. We recently created a  
89 mouse model with a skeletal muscle specific *Bin1* deletion that is viable and faithfully reproduces the  
90 decreased muscle force and most histopathological hallmarks of CNM (*Bin1*<sup>mck-/-</sup>; unpublished). Here,  
91 we focus on omics analysis of *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup>, *Dnm2*<sup>SL/+</sup> mice, since they represent faithful models  
92 for the three canonical CNM forms and the mice share a similar skeletal muscle organization with  
93 patients.  
94 Several therapeutic proof-of-concepts were recently validated in different CNM models, including the  
95 three CNM mice investigated here<sup>17</sup>. Adeno-associated virus (AAV) transduction of MTM1 or its closer  
96 homolog MTMR2 rescued the *Mtm1*<sup>-/-</sup> mouse, and AAV-MTM1 was further validated in the MTM1  
97 Labrador model and recently injected in patients in a clinical trial<sup>31-33</sup>. Downregulation of DNM2 level  
98 were found increased at least in *Mtm1*<sup>-/-</sup> mice and MTM1 patients, whereas normalization of DNM2

level rescued the *MtmI*<sup>-/-</sup> mouse, the *BinI*<sup>-/-</sup> and *BinI*<sup>mck-/-</sup> mice, and both *Dnm2*<sup>RW/+</sup> and *Dnm2*<sup>SL/+</sup> mice<sup>28, 34-36</sup>. DNM2 was reduced through three methods : genetic cross with a *Dnm2*<sup>+/-</sup> mouse, shRNA, or antisense oligonucleotides<sup>34, 37, 38</sup>. Overexpression of BIN1 through genetic cross with a TgBIN1 mouse or AAV-BIN1 expressing human BIN1 rescued *MtmI*<sup>-/-</sup> and *BinI*<sup>-/-</sup> mice<sup>39</sup>. In addition, treatment with tamoxifen, an estrogen modulator already used in clinic for breast cancer, partially rescued the *MtmI*<sup>-/-</sup> mouse, potentially representing a drug repurposing strategy<sup>40, 41</sup>. Additional potential therapies have been tested in mice and other CNM models, and include *Dnm2* allele-specific silencing or trans-splicing, *Pik3c2b* downregulation, mTOR or acetylcholine esterase inhibitors<sup>17</sup>.

To identify the main pathomechanisms, potential biomarkers and novel therapeutic targets for different forms of CNM, we performed transcriptome and proteome analyses of muscles from *MtmI*<sup>-/-</sup>, *BinI*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mice either developing the disease or treated with 3 different therapies including two methodological approaches for one of the targets. We identified disease and therapeutic molecular signatures common to the three main forms of CNM.

## Results

### Animal models, treatments and omics strategies

In mice, muscle embryonic development proceeds from E10.5 to birth that happens at about E19<sup>42</sup>. Then, muscle growth during postnatal maturation follows 2 phases: one based on satellite cells fusion up to 2-3 weeks (w) and a second based on growth factor signaling from weaning (around 3 w of age) to adulthood at 7 w (Figure 1a)<sup>43, 44</sup>. *MtmI*<sup>-/-</sup> mice develop a progressive myopathy from 2-3 w leading to a strong muscle hypotrophy and decreased locomotor activity, and death by about 8 w. *BinI*<sup>mck-/-</sup> mice have near normal locomotor activity and decreased muscle force at 8 w. *Dnm2*<sup>SL/+</sup> mice have some feeding defects at birth correlating with decreased body weight, strong muscle atrophy and decreased locomotor activity, and survive in adulthood. All these mice have CNM-like histopathology by 7-8 w.

123 Based on the key steps of muscle maturation and on the disease progression in the different models, we  
124 performed RNAseq for transcriptome analysis in tibialis anterior (TA) muscle from the 3 CNM mice at  
125 7 w. In addition, RNAseq was done at E18.5 and 2 w for a longitudinal follow-up of *Mtm1<sup>-y</sup>* mice. Mass  
126 spectrometry for proteome analysis was performed in TA at E18.5, 2 w and 7 w for a longitudinal follow-  
127 up in *Mtm1<sup>-y</sup>* mice (Table S1).

128 Several therapeutic approaches were applied to *Mtm1<sup>-y</sup>* mice and consisted of either *BIN1*  
129 overexpression by crossing with *TgBIN1* mice (*Mtm1<sup>-y</sup>TgBIN1*), tamoxifen supplementation in food, or  
130 DNM2 downregulation by crossing with *Dnm2<sup>+/-</sup>* mice (*Mtm1<sup>-y</sup>Dnm2<sup>+/-</sup>*)(Figure 1b; Table S1). DNM2  
131 was also downregulated with another method, systemic injection of antisense oligonucleotides (ASO  
132 *Dnm2*), in *Bin1<sup>mck-/-</sup>* and *Dnm2<sup>SL/+</sup>* mice and compared to injection of PBS or control ASO. All these  
133 treated cohorts were analyzed by RNAseq in TA at 7 w and compared to the above untreated mice. In  
134 addition, RNAseq and mass spectrometry of TA from *Mtm1<sup>-y</sup>Dnm2<sup>+/-</sup>* mice was performed at E18.5, 2  
135 w and 7 w. Muscle samples were obtained from our previous studies reporting therapeutic efficacy<sup>28, 34,</sup>  
136 <sup>39, 40</sup>.

137 In addition, in order to identify dysregulated muscle proteins that are potentially circulating, mass  
138 spectrometry was performed *in sera* from WT mice at 7 w and compared with the above muscle  
139 transcriptome and proteome data.

140

#### 141 **Influence of the genetic and environment backgrounds**

142 To assess the impact of the genetic background and animal housing on the transcriptome, we analyzed  
143 different RNAseq data from different cohorts of the same *Mtm1<sup>-y</sup>* mouse line on different genetic  
144 backgrounds and raised in different animal houses. Four different cohorts were evaluated (Table 1):  
145 cohort MTM1-a was crossed on the 129Pas background and bred in France, cohort MTM1-b was on a  
146 50% 129Pas and 50% C57BL/6N background and raised in the same animal facility in France, cohort

147 MTM1-c was raised in Switzerland on the 129Pas background, and cohort MTM1-d was bred in Canada  
148 on a C57BL/6J background. Muscles used for transcriptomics for the first three cohorts were TA analyzed  
149 at 7 w. The transcriptome for cohort MTM1-d was previously published and done from quadriceps at 5  
150 w<sup>41</sup>. We compared differentially expressed genes between *Mtm1*<sup>-/-</sup> and WT mice for the different  
151 cohorts. The threshold used to define dysregulated genes was set at Log2FC+/-1 and p-value < 0.05. The  
152 number of dysregulated genes in *Mtm1*<sup>-/-</sup> mice ranged from 1275 to 1981 (Figure 2a). A total of 287  
153 genes were found commonly dysregulated across the different cohorts. These genes correspond to the  
154 disease signature following MTM1 loss, and their expression is not impacted by any environmental or  
155 housing parameters, the genetic background, or the muscle analyzed (Table S2). As expected, the most  
156 divergent transcriptome was from cohort 4 that differs from the other cohorts by both the genetic  
157 background and the muscle, as 56% of dysregulated genes are specific to this cohort versus 23 to 33%  
158 for the other cohorts.

159 All the common dysregulated genes followed the same trend in the different cohorts. We found 67  
160 common genes were downregulated and 220 common genes upregulated. Gene ontology (GO) analysis  
161 revealed an enrichment for muscle development and contraction, cell adhesion and immune cells (Figure  
162 2b). The most upregulated protein coding genes were *Sox11*, *Krt18*, *Mt3*, *Msln*, *Hsf2bp* and *Fosl1*, and  
163 the most downregulated were *Mstn*, *Cdh4*, *Edn3*, *Mtm1*, *Ighm*, *Fam19a4*, *Nt5c1a* and *Amd1* (Table 2).  
164 We thus report the disease signature for MTM1-CNM that is independent of the genetic and  
165 environmental backgrounds in mice.

166

### 167 **Conserved disease signatures linked to *MTM1* mutations among species**

168 We next explored the conservation of the disease signature linked to MTM1 loss in different species. In  
169 human (Biceps brachii or quadriceps), Noguchi et al. performed microarray analysis on a set of 4200  
170 genes previously known to be expressed in skeletal muscle from 8 patients with different *MTM1*

171 mutations <sup>45</sup>. 183 genes were significantly dysregulated compared to unaffected individuals. Of note,  
 172 *MTM1* was not reported dysregulated in this study. In dog, Dupont et al. used RNAseq to analyze two  
 173 hindlimb muscles from Labradors lacking *MTM1* <sup>46</sup>. They found 824 and 1122 genes differentially  
 174 expressed in the Biceps femoris and the Vastus lateralis respectively, with 400 genes dysregulated in both  
 175 muscles. Here, we used the 632 genes that we identified in the RNAseq analyses conducted in *Mtm1*<sup>-/-</sup>  
 176 mouse cohorts MTM1-a, MTM1-b and MTM1-c at 7 w (Figure 2a and Table S3).  
 177 Interspecies analysis revealed 5 differentially expressed genes shared by mouse, dog and human (Figure  
 178 2c). Among them, *CHRND* and *CHRNA1* coding for two subunits of the acetylcholine receptor in the  
 179 NMJ were upregulated (Figure 2d). *MYOG* (myogenin), coding for a transcription factor key in muscle  
 180 differentiation was upregulated in dog and mouse and downregulated in human. *POPDC3* was  
 181 upregulated in human and mouse and downregulated in dog, is also implicated in muscle development,  
 182 and was found mutated in limb-girdle muscular dystrophy <sup>47</sup>. The majority of the dysregulated genes  
 183 were specific to each species, therefore we analyzed them by GO enrichment and it revealed a few  
 184 processes that might be species dependent, as hemostasis dysregulation in human or specific impact on  
 185 kinase pathways in dog (Figure S1 and Table S4).  
 186 As the human data was based on microarray analysis of only a subset of genes, additional comparisons  
 187 were done between dogs and mice and identified 63 additional genes dysregulated (Vastus Lateralis vs  
 188 Tibialis anterior) (Figure 2c and Table S5), or 49 genes (Biceps Femoris vs Tibialis anterior)(Figure S1;  
 189 Table S6). GO terms related to these genes were highly enriched in muscle development (Figure 2e).  
 190 Among these genes, *Chrna1*, *Chrnd* and *Chrng* highlight the NMJ, and *Myog* and *Pax7* the  
 191 transcriptional regulation of muscle differentiation and regeneration. Other examples confirmed by RT-  
 192 qPCR included downregulation of *Mstn* (myostatin), a ligand of TGFβ receptor involved in muscle  
 193 growth, and upregulation of *Fst* (follistatin), coding for an inhibitor of myostatin (Figure 2f). We found  
 194 upregulation of *Igfbp2*, coding an insulin-like growth factor-binding protein potentially involved in

195 muscle differentiation and hypertrophy. *Cilp* was upregulated and codes for a regulator of IGF1 (insulin-  
196 like growth factor type 1) and TGF $\beta$  signaling. Genes coding for potential regulators of the Rac1-actin  
197 pathway, *Tiam2* and *Arhgap36*, were also upregulated.

198 Overall, defects in muscle development and the NMJ appear conserved in mouse, dog and human with  
199 MTM1-CNM. The more detailed investigations in mouse and dog highlighted additional pathways of  
200 interest such as muscle growth and repair.

### 201 **Longitudinal molecular profiling of *Mtm1*<sup>-/-</sup> mice through disease progression**

202 Several pathways were identified in the MTM1-CNM disease signatures at an age when mice, dogs and  
203 patients are strongly affected. To define the primary molecular causes versus consequences of the disease,  
204 we performed longitudinal analyses of the transcriptome and proteome of TA muscles from *Mtm1*<sup>-/-</sup> mice  
205 at pre-symptomatic age (E18.5), early (2 w) and late (7 w) disease stages (Figure 1a). We used the cohort  
206 MTM1-a, including untreated *Mtm1*<sup>-/-</sup> mice, *Mtm1*<sup>-/-</sup> mice rescued by *Dnm2* genetic downregulation,  
207 treated and healthy *Mtm1*<sup>-/-</sup>*Dnm2*<sup>+/-</sup> mice, and their WT littermates (Table S1).

208 Principal component analysis (PCA) on transcriptome data showed that age explains most of the variance  
209 between the mouse groups as underlined by the first principal component (PC1 58%, PC2 13%, PC3 5%  
210 variance; Figure 3a and Figure S2). Separation of the genotypes appeared at PC4 (4% variance).  
211 Interestingly, this separation appeared at 2 w and increased at 7 w. No genes were significantly  
212 dysregulated at E18.5, indicating no difference between genotypes at late embryonic stage. A total of  
213 1175 genes were dysregulated at 2 w and 1981 at 7 w (Figure 3b and Table S7). Potential disease causes  
214 found at 2 w are defects of muscle contraction, sarcomere organization and cell adhesion (GO term  
215 analysis; Figure 3b; Figure S3). Disease consequences found only at 7 w highlighted activation of the  
216 inflammation pathway, suggesting infiltration of affected muscles by immune cells. Volcano plots display  
217 the most dysregulated genes at 2 and 7 w (Figure 3c,d). As examples, the three most upregulated genes  
218 at both ages were *Slh* (sarcolipin), *Krt8* and *Krt18* (keratins)(Figure 3c-d). Dysregulation of these and

219 other genes was confirmed by RT-qPCR (Figure S4, S5). The downregulation of *Mstn*, observed in late  
 220 disease stage in dogs and mice, was already apparent at the 2 w early stage in mice (Figure 3e). An  
 221 example for sarcomere organization is *Ahnak2*, whose protein product (AHNAK 2) localizes to Z-line.  
 222 *Tnnt2* (cardiac troponin) and *Myl4* (cardiac myosin light chain) are implicated in muscle contraction,  
 223 expressed in embryonic but not adult skeletal muscle and in adult cardiac muscle under normal  
 224 conditions, and found upregulated in this myopathy. The cell adhesion was represented for example by  
 225 *Itga3* (integrin). Genes underlying the activation of the inflammation pathway at 7 w include *Cxcl1*  
 226 (chemokine) and *Tlr2* (Toll-like receptor).

227 In parallel, PCA was also performed on proteome data and showed that the variance between the mouse  
 228 groups is first explained by age (PC1; 39% variance; Figure 4a) followed by genotypes (PC2; 7%).  
 229 Similarly to the transcriptome data, the genotype separation appeared at 2 w and increased at 7 w,  
 230 indicating no difference at E18.5. Out of 1462 proteins consistently detected in each of the muscle  
 231 samples, 168 proteins were dysregulated at 2 w and 496 at 7 w in the *Mtm1*<sup>-/-</sup> mice compared to WT  
 232 (Figure 4b and Table S8). Potential disease causes found at 2 w were related mainly to muscle sarcomere  
 233 and contraction (GO terms analysis; Figure S6). At 7 w, defects of muscle contraction pathway were  
 234 persistent, and at this late disease stage dysregulation of ribosomal biogenesis (translation) appeared. The  
 235 transcriptome and proteome data analysis consistently highlighted muscle contraction defects as a main  
 236 early sign of the disease. The late dysregulation of ribosomal biogenesis may reflect a compensatory  
 237 mechanism following alteration of protein homeostasis correlated with the strong fiber hypotrophy in  
 238 MTM1-CNM. Examples of dysregulated proteins implicated in muscle contraction include MYH2  
 239 (myosin heavy chain) mutated in a proximal myopathy with ophtalmoplegia (MIM#605337), and  
 240 TNNC1 (troponin) mutated in dilated cardiomyopathy (MIM#611879)(Figure 4c-e). These proteins  
 241 strongly correlate pathways and gene families found through the above transcriptome analysis: MYH2  
 242 and MYL4 are myosin heavy and light chains, respectively and TNNT2 and TNNC1 are troponins. All  
 243 four proteins are implicated in muscle contraction. Pearson correlation analysis did not underline a high

correlation between specific genes and proteins at 2 and 7 w (Figure S7), as generally reported in the literature<sup>48</sup>. In conclusion, while the same dysregulated genes/proteins are not necessarily highlighted by the transcriptome and proteome analyses, the same pathways and functions are consistently defective in early and late disease stages. Dysregulation in muscle contraction appears to be an early defect in the MTM1-CNM pathology in mice.

### **Disease signature common to several CNM forms**

Next, we explored whether a common disease signature can unify the different CNM forms linked to either *MTM1*, *BIN1* or *DNM2* mutations. First, we compared the levels of these genes/proteins in the different corresponding models: the *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mice (Table 3 and Figure S8). Apart from the lack of MTM1 and BIN1 proteins in their respective knockout mice, DNM2 was found slightly elevated (2.2 fold; p=0.057; <sup>28</sup>) in the *Dnm2*<sup>SL/+</sup> mouse. No strong alteration of the level of their RNA was detected in the corresponding RNAseq data. A slight increase in both BIN1 and DNM2 proteins was found in the *Mtm1*<sup>-/-</sup> mice, and DNM2 protein was slightly increased in *Bin1*<sup>mck-/-</sup>.

Then, dysregulated genes were extracted from each individual cohort at 7 w: cohorts MTM1-a, MTM1-b, MTM1-c, cohort BIN1 (*Bin1*<sup>mck-/-</sup>) and cohort DNM2 (*Dnm2*<sup>SL/+</sup>), and compared to their respective WT littermates. For each cohort, disease models were well separated from the WT controls on the principal component 1 of the PCA (Figures 3a, 5a; Figure S9). The number of dysregulated genes correlated with the severity of the related models at this age: more than 1200 genes for the most severe *Mtm1*<sup>-/-</sup> mouse that does not survive beyond 9 w, 780 genes for the *Dnm2*<sup>SL/+</sup> mouse with strong locomotor deficiency, and 308 for the *Bin1*<sup>mck-/-</sup> mouse with a conserved locomotor function. Among the 25494 genes detected in all the different transcriptomes, 155 common dysregulated genes were identified (Figure 5b and Tables S7, S9, S10, S11, S12, S13). The main cellular component GO terms highlighted the NMJ, basement membrane, sarcomere and activation of the inflammation pathway, previously



268 identified as the main pathways dysregulated in the *Mtm1*<sup>-/-</sup> cohorts (Figure 5c). Thus, the main disease  
269 signature common to the three CNM models underlines defects in sarcomere maturation and function,  
270 and in NMJ maturation as main causes of muscle weakness, and alteration in cell adhesion and basement  
271 membrane as a potential explanation for the altered fiber shape. As previously noted for the *Mtm1*<sup>-/-</sup>  
272 cohorts, a potential increase in the inflammation transcriptome supports the infiltration of immune cells  
273 in the different models. Several genes associated with inflammation were shared between the different  
274 CNM forms as indicated by GO terms linked with macrophages (Figure 5c). Since inflammation was not  
275 previously demonstrated in CNM models, we labelled macrophages with an anti-CD68 antibody on TA  
276 muscle sections. We found a significant increase in macrophage infiltration in the 3 CNM mouse models  
277 (Figure 5 d,e).

278 RT-qPCR analyses confirmed the dysregulation of all the 14 genes tested in the three different mouse  
279 models (Figures S10, S11, S12). In particular, the common CNM disease signature encompassed genes  
280 coding for myosin (MYL4) and troponin (TNNT2) for sarcomere organization and contraction,  
281 acetylcholine receptor subunits (CHRNA1, CHRNA9, CHRND), the SOX11 and MYOG transcription  
282 factors, the calcium regulator sarcolipin (SLN), myostatin and follistatin, and several proteins further  
283 studied below (ANXA2, S100A4, CILP, FETUB, SERPINB1A, IGFBP2)(Figure 5f). Genes specifically  
284 dysregulated in each CNM forms have been analyzed by GO enrichment and revealed some specific  
285 features as hemostasis defect in the BIN1 cohort, or cardiac and lipid metabolism for the DNM2 cohort,  
286 which remain to be further explored (Figure S13 and Table S14).

287 It is interesting to note that orthologs of several genes in the common disease signature were previously  
288 associated with neuromuscular diseases: *LMNA* in Emery-Dreifuss muscular dystrophy and *KLHL40* in  
289 nemaline myopathy, *CHRNA1* and *CHRNA9* for myasthenic syndrome, *HSPB1* and *PDK3* for Charcot-  
290 Marie-Tooth peripheral neuropathy. Overall, the main defective pathways found in the MTM1-CNM

models are also altered in the BIN1-CNM and DNM2-CNM models, revealing the existence of a pathomechanism common to most CNM forms.

### **Therapy signature common to several rescuing approaches for different CNM forms**

The transcriptome responses to different treatments of these three CNM mouse models was assessed in TA muscle at 7 w. *MtmI*<sup>-/-</sup> mice were treated with the drug tamoxifen (cohort MTM1-c), or following genetic crosses with mice either overexpressing human BIN1 (cohort MTM1-b; *MtmI*<sup>-/-</sup>TgBIN1) or with *Dnm2* downregulation (cohort MTM1-a; *MtmI*<sup>-/-</sup>*Dnm2*<sup>+/-</sup>)(Figure 1b). A different methodology to reduce DNM2, systemic injection of ASO *Dnm2*, was used to treat *Bin1*<sup>mck-/-</sup> (cohort BIN1) and *Dnm2*<sup>SL/+</sup> (cohort DNM2) mice. All these therapies improved the phenotypes of the different disease models (<sup>28, 34, 39, 40</sup> and unpublished for *Bin1*<sup>mck-/-</sup>). In each cohort, 4 groups were studied: treated (=rescued) and untreated CNM disease models and treated and untreated WT controls.

PCA showed that untreated and treated WT controls cluster together, suggesting treatments had no general effects on WT mice (Figures 6a,3a and Figure S9). For example, only 112 genes were dysregulated in tamoxifen-treated WT mice, while *Dnm2* was indeed found downregulated together with 15 other genes in WT treated with ASO *Dnm2* (Table S12). The transcriptome of tamoxifen treated *MtmI*<sup>-/-</sup> mice was similar to that of diseased *MtmI*<sup>-/-</sup> mice, suggesting that tamoxifen did not have a strong transcriptional effect. Genetic downregulation of *Dnm2* in *MtmI*<sup>-/-</sup>*Dnm2*<sup>+/-</sup> led to a partial rescue of the transcriptome of *MtmI*<sup>-/-</sup> mice. Analyzing in *MtmI*<sup>-/-</sup>*Dnm2*<sup>+/-</sup> mice over time, we found a partial rescue of 255 genes over 1175 genes dysregulated in *MtmI*<sup>-/-</sup> mice at 2 w, and 725 genes over 1981 dysregulated genes at 7 w disease stage (Figure 6b and Table S7). *Myh3*, *Myh8* and *Sln* are among the best normalized genes at 2 w. Acute downregulation of *Dnm2* with ASO *Dnm2* in both *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mice also partially rescued their transcriptomes (Figure 6a). Genetic overexpression of BIN1 in *MtmI*<sup>-/-</sup>TgBIN1 normalized the transcriptome to a WT level. As a metric to compare the molecular efficacy of the different therapies, we calculated the ratio of the number of genes dysregulated in the

315 rescued group versus the disease group over the number of genes dysregulated in the disease group versus  
316 the WT control; in other words, the ratio of the therapy signature over the disease signature. The  
317 percentage of rescued genes was 0.5% for the MTM1-c cohort (tamoxifen), 36% for the MTM1-a cohort,  
318 43% for the DNM2 cohort and 47% for the BIN1 cohort (Dnm2 downregulation), and 96% for the  
319 MTM1-b cohort (BIN1 overexpression; 1680 genes on 1745). Overall, BIN1 overexpression appears to  
320 be the most efficient therapy to normalize the transcriptome defects of CNM in mice.

321 To determine the common therapy signature, we compared the transcriptome of the rescued mice versus  
322 the transcriptome of the diseased mice for all cohorts except the MTM1-c cohort as tamoxifen treatment  
323 had no strong transcriptomic impact. We found 42 genes defining the therapy signature common to all  
324 therapies in all CNM forms and that were retrieved in the disease signature for most of them (Figure 6b  
325 and Tables S9, S15). Comparison of the rescued versus WT transcriptomes identified no genes in  
326 common that were resistant to all different therapies tested. The expression levels of several of these 42  
327 genes was confirmed by RT-qPCR (Figure 6c and Figures S4, S5, S10, S11, S12). Depending on the  
328 cohorts, expression of these genes was partially or fully rescued upon treatment. For example, *Anxa2*  
329 expression was not rescued in the MTM1-a cohort (*Mtm1<sup>-/-</sup>Dnm2<sup>+/-</sup>*), while *Cilp*, *Fetub* and *Igfbp2* were  
330 all dysregulated in the different diseased models and their expression rescued to WT level following any  
331 treatments.

332 The proteins corresponding to these 42 genes of the common therapy signature could represent novel  
333 therapeutic targets. To help pre-selecting the best candidates, we retrieved the 35 corresponding human  
334 orthologs and interrogated a drug database (<http://drugcentral.org>)(Figure 6d). Two proteins (SCN5A,  
335 SBK3) appeared directly targeted by several drugs, like the antiarrhythmic quinidine for the SCN5A  
336 sodium channel, or nintedanib, an inhibitor of SBK3 and tyrosine kinases used for pulmonary fibrosis  
337 and cancer. Other therapeutic targets that were dysregulated in a majority of cohorts include myosins,

troponins, myostatin or acetylcholine receptor subunits, for which *in vivo* modulation methods were already validated for other diseases.

Taken together, comparison of the three CNM models and several therapies underlined a common disease signature and a common therapy signature indicating potential therapeutic targets and biomarkers to follow disease severity or progression and therapy efficacy.

#### **Identification of muscle and circulating biomarkers correlating with disease and therapy**

To identify potential circulating biomarkers of disease state and therapy efficacy, we compared the list of genes in the disease signature and the therapy signature with proteins detected by mass spectrometry on the serum of WT mice at 8 w (Table S16), with public databases listing proteins detected in different fluids in human and mouse (GTEx, BioGPS, Illumina, GXD), and with the literature (Figure 7a). The following proteins were selected: ANXA2, CILP, FETUB, IGFBP2 and MSTN. To identify the best biomarkers, they were further screened by RT-qPCR, western blotting and ELISA in muscle and in plasma. The levels of all these RNAs were altered in the disease state and responded to the therapies in the 3 CNM mouse models, as validated by RT-qPCR in muscle (Figure 6c; Figures S4, S5, S10, S11, S12). ANXA2, FETUB and CILP proteins were found dysregulated in muscle from some or all CNM models by western blot (Figure 7b and Figure S14). For example, *Anxa2* (ANXA2) was significantly upregulated in muscles from 7 w old *Mtm1<sup>-/-</sup>*, *Bin1<sup>mck-/-</sup>* and *Dnm2<sup>SL/+</sup>* mice at both RNA and protein levels, and its RNA was found already upregulated, albeit to a lesser extent, at 2 w in *Mtm1<sup>-/-</sup>* mice (2 fold at 2 w and 2.46 fold at 7 w).

Concerning circulating biomarkers, ELISA assays confirmed the presence of ANXA2, CILP, IGFBP2 and MSTN in the plasma from WT mice, as previously detected in serum by mass spectrometry (Figure 7c). Unlike in muscle, CILP levels in plasma were not changed in any CNM mouse models. Interestingly, the plasma level of IGFBP2 was significantly increased in *Mtm1<sup>-/-</sup>* mice. The alteration of *Igfbp2* RNA

362 levels in the *MtmI*<sup>-y</sup> muscle increased with age and disease progression, from 6 fold at 2 w (early disease  
363 stage) to 17 fold at 7 w (late disease stage). To assess if circulating IGFBP2 is a biomarker common to  
364 several CNM forms, ELISA assays were performed in *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> plasma at 7 w. The level  
365 of IGFBP2 was changed only in *MtmI*<sup>-y</sup> mice, suggesting it could be a biomarker specific to the MTM1-  
366 CNM form. Similarly, dysregulation of plasma protein content of ANXA2 and MSTN was revealed by  
367 ELISA, specifically for the BIN1-CNM or MTM1-CNM models respectively. In both cases, these  
368 biomarkers responded to the therapies. The increase in ANXA2 plasma protein content in *Bin1*<sup>mck-/-</sup> mice  
369 was normalized upon DNM2 decrease with ASO *Dnm2*. The strong decrease in MSTN plasma protein  
370 content in *MtmI*<sup>-y</sup> mice was normalized upon BIN1 overexpression.

371 Overall this screening strategy, from RNAseq based discovery, to RT-qPCR and western blot in muscle,  
372 and to ELISA in plasma, discovered ANXA2 as a muscle biomarker for several CNM forms and CILP  
373 and FETUB for specific CNM forms. IGFBP2, ANXA2 and MSTN were found as circulating biomarkers  
374 for specific CNM forms, and ANXA2 and MSTN plasma levels responded to the therapies.

375

## 376 Discussion

377 We performed a multi-omics meta-analysis of centronuclear myopathies through the comparison of  
378 mouse models for the three main CNM forms and the comparison of three therapies with different targets.  
379 We identified disease signatures for MTM1-CNM conserved in different genetic and environmental  
380 backgrounds and in different species (mouse, dog, human). Longitudinal transcriptome and proteome  
381 analysis of *MtmI*<sup>-y</sup> mice suggested early causal pathomechanisms and late compensatory adjustments.  
382 A disease signature common to the three CNM forms was defined, suggesting a common  
383 pathomechanism for CNM independent of the mutated genes. Comparison of the molecular effect of the  
384 different therapies revealed a correlation between the molecular normalization and the phenotypic rescue.  
385 In addition, novel potential therapeutic targets were suggested. Further molecular and biochemical

386 investigations identified several biomarkers for disease state and therapy efficacy in muscle (RNA,  
387 proteins) and in plasma.

388

389 **Pathomechanism of centronuclear myopathies**

390 We compared the muscle transcriptomes of the *MtmI*<sup>-/-</sup>, *BinI*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mice faithfully  
391 reproducing the muscle weakness and histological hallmarks of the three main CNM forms<sup>28, 49</sup>. The  
392 overall transcriptomes easily distinguished the CNM models from their WT littermates.

393 For MTM1-CNM, to identify the specific disease signature independent from the genetic backgrounds  
394 or environment, we increased data heterogeneity by characterizing several *MtmI*<sup>-/-</sup> groups on 129Pas,  
395 C57BL/6J or mixed backgrounds bred in different animal houses, and then focused on the common  
396 transcriptome dysregulation. Next, this signature was compared to available transcriptome data from one  
397 MTM1 canine model and to a partial microarray analysis (4200 genes) of patient muscle biopsies<sup>45, 46</sup>.

398 Our conclusions were supported by previous findings. Dysregulation of NMJ components (CHRNA1,  
399 CHRND, CHRNG) were recently reported in the MTM1 dog and alteration of NMJ function was  
400 suggested in a *mtmI* knockdown zebrafish and in *MtmI* mouse models<sup>46, 50, 51</sup>. We found a high increase  
401 in *Sln* expression in the three CNM mouse models, and upregulation of *Sln* was previously reported  
402 following microarray analysis of the *MtmI*<sup>-/-</sup> mouse<sup>49</sup>. In addition, longitudinal analyses of the muscle  
403 transcriptome and proteome of *MtmI*<sup>-/-</sup> mice at pre-symptomatic (E18.5), early (2 w) and late (7 w)  
404 disease stages highlighted the same dysregulated pathways, although the same dysregulated  
405 genes/proteins were not necessarily found (Figures 3, 4). These ages were chosen to potentially  
406 distinguish between early causes of the disease and late consequences or compensatory mechanisms.  
407 Further functional investigations are needed to confirm their causality. In addition, the proteome analysis  
408 only covered the most abundant proteins. For example, BIN1 and DNM2 were not detected in the muscle  
409 proteomes. However, BIN1 and DNM2 proteins were both increased in *MtmI*<sup>-/-</sup> mice using specific  
410 antibodies by western blot (Table 3)<sup>34, 39</sup>. Interestingly, we recently showed that the *MtmI*<sup>-/-</sup> mice can be

411 rescued by either decreasing DNM2 or increasing BIN1 <sup>37, 39</sup>, strongly supporting the idea that DNM2  
412 increase is a disease cause while BIN1 increase is a compensatory mechanism.

413 The comparison of the *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> muscle transcriptomes revealed a common  
414 disease signature, encompassing sarcomere organization, muscle contraction, muscle development and  
415 cell adhesion. All these pathways were already the main ones found dysregulated at 2 w in *Mtm1*<sup>-/-</sup> mice,  
416 supporting the hypothesis that their dysregulation represents the main pathomechanism for all CNM  
417 forms (Figures 3, 4, 5). In addition to these pathways, inflammation activation (transcriptome) and  
418 ribosomal biogenesis (proteome) were found only at 7 w suggesting that their dysregulation is a  
419 consequence or a response to the disease state. Based on these data and on the knowledge that the three  
420 CNM proteins regulate membrane remodeling, we propose a model for the pathomechanism of CNM.

421 Alteration of the triad membrane structure would lead to impaired calcium signaling and defective  
422 muscle contraction, explaining the strong muscle weakness and hypotonia seen in patients. The myofiber  
423 hypotrophy seen in patients and mouse models correlates with alteration of muscle development and  
424 regeneration RNA markers, and may be related to dysregulation of IGF modulators as IGFBP2 or/and to  
425 the reported decrease in satellite cells in patients <sup>52</sup>. The strong myofiber hypotrophy would then trigger  
426 a later adaptation on protein homeostasis, as underlined by the increase of the ribosome biogenesis genes  
427 found only at 7 w. Indeed, defects of protein homeostasis correlate with the alteration of autophagy and  
428 the ubiquitin-proteasome pathways found in *Mtm1*<sup>-/-</sup> mice <sup>25, 53, 54</sup>. In parallel, a primary defect in cell  
429 adhesion would impact the basement membrane and mechanotransduction and may explain defects in  
430 muscle contraction and also the altered fiber shape found in patients and mice. This is in agreement with  
431 the accumulation of integrins in *Mtm1*<sup>-/-</sup> mouse and patient myofibers, and with the proposed link  
432 between MTM1 and beta integrin recycling <sup>39, 55, 56</sup>. The observed increased in interfiber space, together  
433 with the alteration of muscle regeneration, would cause the late increase in genes implicated in  
434 inflammation activation. As we detected a significant increase in RNA markers of inflammatory cells  
435 while performing whole tissue RNAseq, we conclude there is a significant infiltration of inflammatory

436 cells that was confirmed by immunofluorescence labelling and quantification (Figure 5d-e). An  
 437 inflammatory component was not previously reported for CNM but is common in dystrophies <sup>57</sup>.  
 438 Furthermore, the dysregulated pathways found here were barely underlined previously in the other CNM  
 439 forms linked to *BINI* or *DNM*. Overall, although some of these pathways were previously found altered  
 440 in MTM1-CNM, these omics analyses allowed to obtain a more complete and detailed overview of the  
 441 pathomechanisms and extend it to several other CNMs. The GO term analysis of the common disease  
 442 signature identified here for different CNM forms mostly reflects general muscle dysfunction and  
 443 compensatory mechanisms put in place by the myofibers to cope with these defects, and thus highlights  
 444 pathways also dysregulated in a large number of muscle diseases. However, differences appear when  
 445 looking at the gene level that may reveal plasticity to impact or compensate the main muscle pathways  
 446 depending on the sub-class of myopathies and on the primary genetic defect.

447 To a greater extent, we found several genes mutated in different neuromuscular diseases in the common  
 448 CNM disease signature. Notably, *LMNA* is mutated in Emery-Dreifuss muscular dystrophy and the  
 449 encoded protein lamin A/C regulates nuclear envelope stability (MIM#181350, 616516) <sup>58</sup>. Of note,  
 450 lamin A/C and BIN1 both bind the LINC complex that regulates nuclear shape and positioning and BIN1-  
 451 CNM patients have an altered nuclear envelope structure <sup>59</sup>. *KLHL40* is mutated in another congenital  
 452 myopathy and is a substrate adaptor for the E3-ubiquitin ligase Cullin-3 (MIM#615348) <sup>60,61</sup>. Similarly,  
 453 MTM1 binds the Cullin-3 partner UBQLN2 and MTM1-CNM is linked to defects in the ubiquitin-  
 454 proteasome pathway <sup>54</sup>. Several dysregulated genes in all CNM models are mutated in cardiomyopathy :  
 455 *SCN5A* (MIM#601154), *TNNT2* (MIM#601494) and *MYL4* (MIM#617280). In addition, MYH2 and  
 456 TNNC1 found upregulated in the proteome of 2 w *Mtm1*<sup>-/-</sup> mice are also mutated in a proximal myopathy  
 457 (MIM#605637) or a cardiomyopathy (MIM#611879), respectively. Such findings are commonly  
 458 observed in myopathies where upregulation of genes usually expressed in embryonic muscle or adult  
 459 cardiac muscle are re-expressed in the affected skeletal muscle. Finally, *CHRNA1* (MIM#601462) and



460 *CHRND* (MIM#616322) are mutated in congenital myasthenic syndromes, correlating with the defect in  
461 NMJ found in MTM1-CNM models<sup>50, 51, 62</sup>.

462

### 463 **Common therapeutic targets for centronuclear myopathies**

464 We compared here three therapies involving three different targets in MTM1-CNM models, BIN1  
465 overexpression, tamoxifen treatment and DNM2 regulation, and two methodologies for the latter target.  
466 For the first time, this allows a molecular comparison of the different therapies for CNM. BIN1  
467 overexpression appears to be the most efficient therapy to normalize the molecular defects. The  
468 percentage of rescued genes varies greatly with 96% for BIN1 overexpression, 36% for DNM2  
469 downregulation and 0.5% for tamoxifen in the MTM1-CNM mouse model. In the AAV-MTM1 treated  
470 dogs, the percentage of rescued genes was 52% and 43% depending on the muscles analyzed<sup>46</sup>. These  
471 findings highlight a correlation between the phenotypic and molecular rescue, as modulations of either  
472 MTM1, BIN1 or DNM2 improved the lifespan, locomotor and histological phenotypes very efficiently,  
473 while tamoxifen treatment resulted in a partial increase in lifespan and a significant amelioration of the  
474 histopathology, although to a lesser extent than with genetic crosses<sup>17, 31, 34, 37, 39-41</sup>. As a potential  
475 explanation to the different rescue efficiency of the transcriptome dysregulation, BIN1 may directly  
476 modulate the general transcription program in a disease context, while MTM1 and DNM2 may directly  
477 impact on the cellular (proteins, membrane) defects. Indeed, BIN1 binds the transcription factor MYC  
478 and can shuttle between the cytoplasm and the nucleus in muscle cells<sup>63-65</sup>. Of note, BIN1 overexpression  
479 was achieved through genetic cross and is thus chronic from embryogenesis, while AAV-MTM1 and  
480 tamoxifen treatments are postnatal. However, transcriptomes comparison between chronic (*Dnm2*<sup>+/-</sup>  
481 genetic cross for MTM1-a) and acute DNM2 downregulation (ASO *Dnm2* injection for DNM2 and BIN1  
482 cohorts) showed similar percentages of rescued genes: 36% in MTM1-a, 43% in DNM2 and 47% in  
483 BIN1 cohorts (Figure 6). The rescuing effect of tamoxifen treatment is not based on transcriptome  
484 remodeling but might directly involve membrane and protein functions.

485 We evaluated the toxicity of the different therapies used in this study by comparing the WT treated versus  
486 WT mice. Treatment of WT mice did not show a strong impact on the transcriptomes, while it was not  
487 reported in AAV-MTM1 treated dogs. For example, injection of ASO *Dnm2* decreased *Dnm2* expression  
488 but had few off-targets. No detectable phenotypic toxicity were observed in the treated WT mice.  
489 However, in human, even if the same gene will be targeted (either BIN1 or DNM2), the therapeutic  
490 compound and formulation may differ and the delivery method and corresponding dose will change.  
491 Here, for BIN1 overexpression we used the human cDNA while for DNM2 downregulation we used  
492 antisense oligonucleotides specific to the mouse *Dnm2* gene. We also detected the expected  
493 overexpression of the human *BIN1* gene in the MTM1-b cohort. BIN1 overexpression only changed the  
494 expression of three genes in the treated WT mice (Figure S9), while rescuing most transcriptome  
495 dysregulation in the *Mtm1*<sup>-/-</sup> mice, suggesting this therapy modulates the transcriptome mainly in a  
496 disease context (i.e. normalization).

497 The present data revealed several potential targets that were not directly targeted in the experiments (i.e.  
498 not MTM1, BIN1 nor DNM2). Genes of interest are expected to be dysregulated in disease and  
499 normalized upon efficient therapies, i.e. part of the therapy signature. In addition, known drugs targeting  
500 these gene products may be an asset, as it will allow drug repurposing and a faster clinical development.  
501 Several genes coding for subunits of the acetylcholine receptor are dysregulated. In particular  
502 acetylcholine esterase inhibitors used in clinical trial to treat myasthenic syndromes were tested with  
503 some success in different CNM forms <sup>62</sup>. *Scn5a* is also found upregulated in the disease signature and  
504 normalized in the therapy signature, and encodes a subunit of the sodium channel that can be inhibited  
505 by quinidine, a stereoisomer of quinine. *Mstn* encoding the myokine myostatin, an inhibitor of muscle  
506 growth, is significantly decreased in disease models of all cohorts except the *Bin1*<sup>mck-/-</sup> mouse (Log2FC  
507 = -0.6 while our threshold was -1). Inhibitors of myostatin are being tested in the clinic for other muscle  
508 diseases, and one of them (ActRIIB-mFc) showed only a very mild amelioration of the *Mtm1*<sup>RC/y</sup> knock-  
509 in mouse <sup>66</sup>. The mild amelioration can be explained by the fact that myostatin is already strongly

510 decreased in the disease state and thus could hardly be better suppressed <sup>67</sup>. *Sln* encoding the calcium  
511 regulator sarcolipin is strongly upregulated in disease models of all cohorts except the MTM1-c cohort.  
512 Reducing sarcolipin expression through genetic cross or shRNA improved Duchenne muscular  
513 dystrophy phenotypes in mice <sup>68</sup>. As a last example, myosins and troponins also meet these criteria and  
514 are targeted by several pharmacological regulators that may improve the muscle contraction defects of  
515 CNM. Indeed, all discussed modulations should be first validated in laboratory models, as it is unclear if  
516 dysregulation of some pathways are disease causing or compensatory.

517

### 518 **Potential biomarkers for disease progression and therapy efficacy**

519 We developed a strategy to identify potential biomarkers, combining RNAseq, RT-qPCR and western  
520 blotting in muscle with mass spectrometry and ELISA assays in blood, supported by database and  
521 literature mining. The validity of these biomarkers for monitoring disease progression and therapy  
522 efficacy has to be confirmed in human samples. This will require muscle and blood sampling of untreated  
523 and treated patients with different CNM forms together with adequate aged-matched controls. We found  
524 that *Mstn* RNA level is strongly decreased in *Mtm1*<sup>-/-</sup> and *Dnm2*<sup>SL/+</sup> mice and to a lesser extent in *Bin1*<sup>mck-</sup>  
525 <sup>-/-</sup> (Log2FC=-0.6), and was normalized upon modulation of *MTM1*, *BIN1* and *Dnm2* (Figures S5, S9, S10,  
526 S11) <sup>46</sup>. In agreement, MSTN was recently found decreased in plasma from MTM1- and DNM2-CNM  
527 patients and responded to ASO *Dnm2* treatment in *Mtm1*<sup>-/-</sup> mice <sup>69</sup>. In addition, MSTN plasma level was  
528 also normalized upon BIN1 overexpression (Figure 7c). ANXA2 is a calcium-dependent phospholipid-  
529 binding protein that has a role in muscle repair <sup>70</sup>, and was validated here as a muscle biomarker for all  
530 the CNM forms that we have tested. ANXA2 was detected in plasma, increased in the *Bin1*<sup>mck-/-</sup> mouse  
531 model, and normalized upon ASO *Dnm2* injection. Moreover, several reports cited ANXA2 to be a  
532 valuable biomarker in different cancers <sup>71, 72</sup>.

533 In particular, these potential biomarkers could be used in clinical trials to monitor the  
534 progression/reversion of the disease or/and efficacy of the therapy. Currently, there are two clinical trials

ongoing to treat CNM. Firstly, the clinical trial (NCT03199469) for X-linked myotubular/centronuclear myopathy in patient under 5 years old based on *MTM1* gene replacement using AAV. Secondly, a clinical trial based on the decrease/normalization of *DNM2* with ASO (DYN101) is ongoing in patients with mutations in *MTM1* and *DNM2* over 16 years old (NCT04033159) and is planned for patients between 2 and 17 years old (NCT04743557).

540

## 541 **Conclusions**

Here, we report the first multi-omics analysis of animal models for several CNM forms, and of the effect of different therapies allowing to reveal a common disease signature and a common therapy signature. We determined the global pathological mechanism and deciphered the molecular impact of therapies. Longitudinal analyses of the treated and untreated MTM1-CNM model highlight potential causes and consequences of the pathology. In addition, we identified several novel biomarkers detectable in muscle and/or plasma through different validated methodologies. These findings and the associated data should be an asset to the community for further investigations. More generally, this study validates the concept of using omics to identify molecular signatures common to different disease forms or to several therapeutic strategies.

551

## 552 **Materials and Methods**

### 553 **Animals**

In this study we used different cohorts of mice. The sample size is given in Table S1. The cohort MTM1-a (WT, *Dnm2*<sup>+/-</sup>, *Mtm1*<sup>-/-</sup>, and *Mtm1*<sup>-/-</sup>*Dnm2*<sup>+/-</sup>) was previously phenotyped on a 129Pas genetic background <sup>34</sup>. The cohort MTM1-b (WT, *TgBIN1*, *Mtm1*<sup>-/-</sup>, and *Mtm1*<sup>-/-</sup>*TgBIN1*) was previously phenotyped on a 50% 129Pas and 50% C57BL/6N genetic background <sup>39</sup>. Both cohorts MTM1-a and MTM1-b were bred in IGBMC animal house in France. The cohort MTM1-c (WT, WT + tamoxifen, *Mtm1*<sup>-/-</sup>, and *Mtm1*<sup>-/-</sup> + tamoxifen) was previously treated and phenotyped on a 129Pas genetic

background<sup>40</sup>. Tamoxifen was administered via supplement pellets of diet (30mg/kg of tamoxifen). This cohort was bred in the animal house of the School of Pharmaceutical Sciences of the University of Geneva, Switzerland. The cohort DNM2 (WT + ASO Ctrl, WT + ASO *Dnm2*, *Dnm2*<sup>SL/+</sup> + ASO Ctrl, *Dnm2*<sup>SL/+</sup> + ASO *Dnm2*) was previously treated and phenotyped on a C57BL/6N genetic background<sup>28</sup>. The cohort BIN1 (WT + PBS, WT + ASO *Dnm2*, *Bin1*<sup>mck/-</sup> + PBS, *Bin1*<sup>mck/-</sup> + ASO *Dnm2*) was previously treated and phenotyped on a C57BL/6N genetic background (unpublished). Both DNM2 and BIN1 cohorts were bred in IGBMC animal house in France. DNM2 and BIN1 cohorts were treated weekly from 3 to 7 weeks of age with intraperitoneal injections of 25mg/kg of ASO (IONIS Pharmaceuticals) targeting *Dnm2*. Only males were analyzed in this study as only *Mtm1*<sup>-/-</sup> males but not *Mtm1*<sup>+/-</sup> females are affected. TA muscles were dissected at E18.5, 2w or 7w and obtained from the previous studies. Data from MTM1-d cohort (WT, *Mtm1*<sup>-/-</sup>) was retrieved from Maani *et al.*<sup>41</sup>. This cohort was analyzed on a C57BL/6J genetic background and bred in University of Toronto animal house in Canada. Quadriceps muscle from 5 w old animals were considered.

573

#### 574 **Blood collection**

575 To collect plasma, blood samples were collected on EDTA-coated tubes (Microvette 500 K3E, Sarstedt) by mandibular puncture. Samples were then centrifuged at +4°C during 10 min at 2,000 x g. To collect serum, mandibular puncture was performed on mice. Blood was collected in a sterile empty tube and kept for 30 min. After coagulation, only the supernatant (serum) was kept for further analysis. Plasma and serum samples were stored at -80°C.

580

#### 581 **RNA extraction and RNAseq**

582 RNA was extracted from Tibialis anterior muscles using TRI Reagent (Molecular Research Center, Cincinnati, USA). RNA sequencing libraries were prepared using the TruSeq Stranded mRNA Sample

584 Preparation Kit and polyA selection and sequenced on a Hiseq4000 as single-end 50 bp reads for cohort  
585 MTM1-a, MTM1-b, MTMT1-c, cohort DNM2 and cohort BIN1.

586

587 **Transcriptome analysis**

588 Reads were preprocessed using cutadapt (version 1.10) in order to remove adapter, polyA and low-quality  
589 sequences (Phred quality score below 20). Reads shorter than 40 bases were excluded from further  
590 analysis. Reads were mapped to ERCC spike sequences (External RNA Controls Consortium) using  
591 bowtie version 2.2.8 and reads mapping to spike sequences were excluded from further analysis. Reads  
592 were mapped onto the mm10 assembly of *Mus musculus* genome using STAR version 2.5.3. Gene  
593 expression quantification was performed from uniquely aligned reads using htseq-count version 0.6.1p1,  
594 with annotations from Ensembl version 96 and union mode. Count tables were analyzed by the open-  
595 source RStudio environment for R and the Bioconductor software. DESeq2 package (version 1.16.1) was  
596 used to normalize, fit and compare the data between groups. Cutoff values for differential expressed  
597 genes determination were as follows: adjusted p-value < 0.05 and absolute value of Log 2 Fold Change >  
598 1. This pipeline was used for cohort MTM1-a, MTM1-b, MTM1-c, DNM2, and BIN1.

599 To determine rescued genes, we developed a metric that quantifies the status of a diseased gene after the  
600 therapy. A diseased gene is defined as dysregulated in the comparison Disease *versus* WT (absolute value  
601 of Log 2 Fold Change > 1 & adjusted p-value < 0.05). The metric is calculated as the ratio between the  
602 Log 2 Fold Change of the two comparisons: Rescues *versus* Disease over Disease *versus* WT. We  
603 stratified the rescued genes into different categories: excessive rescue (metric > 120), not rescued (0 <  
604 metric < 30), partially rescued (30 < metric < 80), rescued (80 < metric < 120) and worsened (metric <  
605 0) (Figure S15).

606 **Quantitative RT-PCR**

607 Synthesis of cDNA was performed with Superscript<sup>TM</sup> IV Transcriptase (ThermoFischer Scientific,  
608 Whaltham, USA). Quantitative PCR was done in a Lightcycler<sup>®</sup> 480 (Roche Diagnostics, Basel,

Switzerland) with SYBR Green Master Mix I (Roche Diagnostics, Basel, Switzerland), and 0.5  $\mu$ M of forward and reverse primers. Primers were validated by amplicon sequencing and melting curve analysis and listed in Table S17. *Stau1*, *Rps11* and *Rpl27* were used as housekeeping genes to normalize gene expression.

613

#### 614 **Protein extraction and Liquid digestion**

TA muscles were lysed in RIPA buffer supplemented with 1 mM PMSF, 1 mM DTT and complete mini-EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). DC Protein Assay Kit (BioRad, Hercules, USA) was used to determine protein concentration. For serum analysis, most abundant serum proteins were depleted with the Proteome purify 2 kit (MIDR002-020, R&D Systems) according to manufacturer instructions before analysis by LC-MS/MS.

Protein mixtures were TCA-precipitated overnight at 4°C. Samples were then centrifuged at 14000 rpm for 30 min at 4°C. Pellet were washed twice with 1 mL cold acetone and centrifuged at 14000 rpm for 10 min at 4°C. Washed pellets were then urea-denatured with 8 M urea in Tris-HCl 0.1 mM, reduced with 5 mM TCEP for 30 min, and then alkylated with 10 mM iodoacetamide for 30 min in the dark. Both reduction and alkylation were performed at room temperature and under agitation (850 rpm). Double digestion was performed with endoproteinase Lys-C (Wako) at a ratio 1/100 (enzyme/proteins) in 8 M urea for 4h, followed by an overnight modified trypsin digestion (Promega) at a ratio 1/100 (enzyme/proteins) in 2 M urea. Both Lys-C and Trypsin digestions were performed at 37°C. Peptide mixtures were then desalted on C18 spin-column and dried on Speed-Vacuum before LC-MS/MS analysis.

630

#### 631 **LC-MS/MS Analysis**

Samples were analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific, San Jose California) coupled in line with a LTQ-Orbitrap ELITE mass spectrometer via a nano-electrospray ionization source

634 (Thermo Scientific, San Jose California). Peptide mixtures were loaded on a C18 Acclaim PepMap100  
635 trap-column (75  $\mu$ m ID x 2 cm, 3  $\mu$ m, 100Å, Thermo Fisher Scientific) for 3.5 min at 5  $\mu$ L/min with 2%  
636 ACN, 0.1% FA in H<sub>2</sub>O and then separated on a C18 Accucore nano-column (75  $\mu$ m ID x 50 cm, 2.6  $\mu$ m,  
637 150Å, Thermo Fisher Scientific) with a 90 min linear gradient from 5% to 35% buffer B (A: 0.1% FA in  
638 H<sub>2</sub>O / B: 99% ACN, 0.1% FA in H<sub>2</sub>O), then a 20 min linear gradient from 35% to 80% buffer B, followed  
639 with 5 min at 99% B and 5 min of regeneration at 5% B. The total duration was set to 120 min at a flow  
640 rate of 200 nL/min. The oven temperature was kept constant at 38°C.

641 The mass spectrometer was operated in positive ionization mode, in data-dependent mode with survey  
642 scans from m/z 350-1500 acquired in the Orbitrap at a resolution of 120,000 at m/z 400. The 20 most  
643 intense peaks (TOP20) from survey scans were selected for further fragmentation in the Linear Ion Trap  
644 with an isolation window of 2.0 Da and were fragmented by CID with normalized collision energy of  
645 35%. Unassigned and single charged states were rejected.

646 The Ion Target Value for the survey scans (in the Orbitrap) and the MS2 mode (in the Linear Ion Trap)  
647 were set to 1E6 and 5E3 respectively and the maximum injection time was set to 100 msec for both scan  
648 modes. Dynamic exclusion was used. Exclusion duration was set to 20 sec, repeat count was set to 1 and  
649 exclusion mass width was  $\pm$  10 ppm.

650

## 651 **Proteome analysis**

652 Proteins were identified by database searching using Maxquant 1.6.6.0 and *Mus musculus* database  
653 (Uniprot Proteome database). Oxidation (M) was set as variable modification, and  
654 Carbamidomethylation (C) as fixed modification. Peptides were filtered with a false discovery rate  
655 (FDR) at 1%, the label-free quantitative values were processed using Perseus 1.6.6.0. 3521 proteins were  
656 identified. Statistical analyses were conducted in R-bioconductor (R-3.6.3). wrMisc, wrProteo packages  
657 were used to normalize, and to impute missing data with default parameters. Cutoff values for differential



expressed protein determination were as follows: adjusted p-value < 0.05 and absolute value Log 2 Fold Change > 1.

**Western Blotting**

Denaturation was performed on samples during 5 min at 95 °C with 5X Lane Reducing Buffer (ThermoFischer Scientific, Whaltham, USA) and loaded on 10% SDS-PAGE gel (161-0173, TGX Fast Cast Acrylamide kit, BioRad). Proteins were transferred to a nitrocellulose membrane using Transblot® Turbo™ RTA Transfer Kit (BioRad, Hercules, USA). Loading was controlled by Ponceau S (P7170, Sigma-Aldrich) staining and Cy5 dye fluorophore (RPN4000, QuickStain). Membranes were blocked 1hr with 5% non-fat dry milk in 0.1% TBS Tween 20 prior to incubations with primary and secondary antibodies. The primary and secondary antibodies used were: ANXA2 (Mouse, 1:1000, sc-28385), CILP-1 (Rabbit, 1:1000, orb182643), FETUB (Rabbit, 1:500, orb252830), MTM1 (2827, 1:700, homemade [34]), BIN1 (R2405, 1:700, homemade [39]), DNM2 (DNM2-R2865, 1:500, homemade [34]), β-Actin (Mouse, 1:5000, homemade), peroxidase-coupled goat anti-rabbit (Goat, 1:10000, 112-036-062) and peroxidase-coupled goat anti-mouse (Goat, 1:10000, 115-036-068).

**ELISA assays**

Plasma proteins were quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins (ANXA2, IGFBP2, CILP, MSTN) were quantified, using ANXA2 ELISA Kit (LS-F5798, LSBio), IGFBP2 ELISA Kit (ab207615, Abcam), CILP ELISA Kit (ABIN5591836, Antibodies-online), MSTN ELISA Kit (DGDF80, RD Systems) respectively, according to the manufacturer's instructions.

**Muscle immunofluorescence**

Transverse cryosections of TA muscles (8µm) were permeabilized with Triton 0.5%, blocked in BSA 5% and incubated overnight at +4°C with anti-CD68 (MCA1957GA BioRad, 1:100) for identifying

683 macrophages, DAPI for staining nuclei and wheat germ agglutinin (WGA) conjugated to alexa fluor AF-  
684 647 for labeling the extracellular matrix. Slides were incubated with an anti-mouse secondary antibodies  
685 for 1h at room temperature (A-11007 Thermo Fisher Scientific, 1:250), observed and photographed in  
686 Leica DM 4000 Bx microscope. The images were analyzed using Image J software ( $n = 3$  mice per  
687 group).

688

### 689 **Orthologues retrieval and gene ontology analysis**

690 Orthologues between mice and dogs, human and mice were retrieved by bitr function in ClusterProfiler  
691 package. Gene ontology analyses were performed with ClusterProfiler package (version 3.12.0) using  
692 the overrepresentation test and the Benjamini–Hochberg correction for multiple testing. Enrichments  
693 with a corrected p-value lower than 0.05 were considered significant <sup>73</sup>.

694

### 695 **Data representation and statistical analyses**

696 PCA, volcano plot, qPCR results were generated in R-bioconductor (R-3.6.3). PCA was generated from  
697 DESeq2 package (version 1.24.0) with variance stabilizing transformation. All genes were used to  
698 generate the PCA from figures 3a and 4a. Disease signature genes (Table S9) were used to generate the  
699 PCA from figure 5a. Therapy signature genes (Table S15) were used to generate the PCA from figure 6b.  
700 Statistical analyses for RT-qPCR were performed by Dunn’s multiple comparison test. Western blot and  
701 ELISA results were analyzed in GraphPad Prism (v.9), using Student test. Venn diagrams were obtained  
702 from InteractiVenn website ([www.interactivenn.net](http://www.interactivenn.net))<sup>74</sup>.

703

### 704 **Data availability**

705 The R script used to process the data has been deposited in GitLab and is freely available at  
706 <http://git.lbgi.fr/djeddi/Myomics>. RNA-sequencing data were deposited in NCBI GEO with the  
707 accession code GSE160084. The mass spectrometry proteomics data have been deposited to the

708 ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021725.  
709 The mass spectrometry proteomics data from the circulating proteins in serum, have been deposited to  
710 the ProteomeXchange Consortium via the PRIDE partner repository with the dataset  
711 identifier PXD021765.

712

713 **Acknowledgements**

714 We thank Luc Negroni, Bastien Morlet, Kirsley Chennen, Matthieu Jung for help in experiments and  
715 analyses, Genomeast for RNAseq, IGBMC platforms of proteomics and animal housing.

716

717 **Authors' contributions**

718 JL designed and supervised the study. SDjeddi performed most bioinformatics analyses with help from  
719 WR, CKeime and JT. SF, CKretz and OD provided mouse samples. DR, AM, SF and ASS performed  
720 RNA quantification. DR, SDjerroud and XMM performed western blots. DR performed ELISA assays.  
721 JDCN performed immunofluorescence. JL and SDjeddi wrote the manuscript.

722

723 **Abbreviations**

- 724 - AAV: Adeno-associated virus
- 725 - ASO: Antisense Oligonucleotides
- 726 - CNM: Centronuclear myopathies
- 727 - ELISA: Enzyme-linked Immunosorbent Assay
- 728 - GO: Gene Ontology
- 729 - IGF: Insulin Growth Factor
- 730 - Log2FC: Log2 Fold Change
- 731 - NMJ: Neuromuscular Junction
- 732 - PCA: Principal Component analysis

733 - PC: Principal Component

734 - RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction

735 - TA: Tibialis anterior

736

## 737 **Competing interests**

738 J.Laporte is co-founder of Dynacure (Illkirch, France).

739

## 740 **Funding**

741 This study was supported by institute funding from Institut National de la Santé et de la Recherche  
742 Médicale, Centre National de la Recherche Scientifique, Université de Strasbourg and by grants from the  
743 Agence Nationale de la Recherche ANR-10-LABX-0030-INRT, a French State fund managed by the  
744 Agence Nationale de la Recherche under the frame program Investissements d'Avenir ANR-10-IDEX-  
745 0002-02, Muscular Dystrophy Association (576154), Fondation pour la Recherche Médicale  
746 (201903007992), and Association Française contre les Myopathies-Téléthon (#20959 to OMD and JL  
747 and #22734 to JL).

748

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## Figure Legends

**Figure 1.** Experimental design. **(a)** Timeline of the different steps occurring during muscle development in mice. The green arrow represents the normal lifespan of control mice (WT). The phenotype of the three mouse models (*Mtm1<sup>-ly</sup>*, *Bin1<sup>mck/-</sup>*, *Dnm2<sup>SL/+</sup>*) used in this study is illustrated by the colored arrows with a color gradient ranging from green for non-affected mice, yellow for the onset of myopathy and red for affected mice. **(b)** Molecular analyses were performed on different mouse models, *Mtm1<sup>-ly</sup>*,



951 *Bin1<sup>mck/-</sup>*, *Dnm2<sup>SL/+</sup>*, untreated or treated by different therapeutic approaches (overexpression of *BIN1*,  
 952 tamoxifen supplementation or downregulation of *Dnm2* either by genetic cross or by ASO injection).  
 953 Disease signature refers to the common dysregulated genes in the three mouse models compared to WT  
 954 littermates, while the therapy signature refers to the common genes rescued following the different  
 955 treatments.

956

957 **Figure 2.** MTM1-CNM signature in different species. **(a)** Venn diagram illustrating the shared  
 958 dysregulated genes based on the *Mtm1<sup>-/-</sup>* vs WT comparison of four mouse cohorts. The percentages of  
 959 uniquely dysregulated genes and the number of differentially expressed genes in each cohort are  
 960 indicated in brackets. **(b)** Gene Ontology (GO) enrichment analysis of differentially expressed genes  
 961 common to the four *Mtm1<sup>-/-</sup>* mouse cohorts. The 20 GO biological process terms with the lowest p-value  
 962 are displayed. **(c)** Venn diagram illustrating the shared dysregulated genes based on MTM1 vs control  
 963 comparison in three different species: human, mice (TA) and dog (*Vastus lateralis*). **(d)** mRNA  
 964 log2foldchange expression of differentially expressed genes common to the three species. **(e)** GO  
 965 enrichment analysis of differentially expressed genes common to mice and dog. The 20 GO biological  
 966 process terms with the lowest p-value are displayed. **(f)** mRNA log2foldchange expression of  
 967 differentially expressed genes common between mice and dog determined by RNAseq (dog, mice) and  
 968 RT-qPCR (mice).

969

970 **Figure 3.** Longitudinal mRNA profiling of *Mtm1<sup>-/-</sup>* mice. **(a)** Principal component analysis of RNAseq  
 971 data. The first and fourth axes are represented. Colored symbols represent genotypes and shapes represent  
 972 ages for each mouse. **(b)** Venn diagram illustrating the shared and specific dysregulated genes based on  
 973 the *Mtm1<sup>-/-</sup>* vs WT comparison at 2 and 7 weeks. The most enriched GO biological processes are  
 974 represented by dashed boxes. **(c)** Volcano plots representing the differentially expressed genes at 2w and  
 975 **(d)** at 7w. Up-regulated genes are in red and down-regulated genes are in blue. **(e)** Gene expression data

976 (log normalized counts) determined by RNASeq for *Mstn* (muscle growth), *Sln* (calcium homeostasis),  
977 *Ahnak2* (sarcomere organization), *Myl4* and *Tnnt2* (muscle contraction), *Itga3* (cell adhesion), *Cx3c1l*  
978 and *Tlr2* (inflammation pathway) across time. Each dot represents an individual mouse, the shaded area  
979 represents the confidence interval at 0.95.

980

981 **Figure 4.** Longitudinal protein profiling of *MtmI*<sup>-/-</sup> mice. **(a)** Principal component analysis of mass  
982 spectrometry data. Colored symbols represent genotypes and shapes represent ages for each mouse.  
983 Technical and biological replicates are shown. **(b)** Venn diagram illustrating the shared and specific  
984 dysregulated proteins based on the *MtmI*<sup>-/-</sup> vs WT comparison at 2 and 7 weeks; the most enriched GO  
985 biological processes are represented by dashed boxes. **(c)** Volcano plots displaying the differentially  
986 expressed proteins at 2w and **(d)** at 7w. Up-regulated proteins are in red and down-regulated proteins are  
987 in blue. **(e)** MYH2 and TNNC1 (muscle contraction), and RPL3 (ribosomal biogenesis) expression data  
988 obtained by mass spectrometry across time. Each dot represents technical and biological replicates, the  
989 shaded areas represent the confidence interval at 0.95.

990

991 **Figure 5.** CNM disease signature in mouse. **(a)** PCA on RNAseq data on the 155 genes commonly  
992 differentially expressed between the five cohorts, each dot represents a mouse. The three different CNM  
993 mouse models are represented by red, orange, yellow dots and the WT controls by green dots. **(b)** Venn  
994 diagram illustrating the shared and specific dysregulated genes between the five CNM cohorts. **(c)** GO  
995 enrichment analysis for biological processes (BP), cellular component (CC) and molecular function (MF)  
996 of the 155 common differentially expressed genes between the five CNM cohorts. GO terms with highest  
997 ratio and lowest q-value are represented. The ratio represents the number of genes dysregulated divided  
998 by the total number of genes in the category. The color scale is based on the q-value, dark colors indicate  
999 most significantly over-represented terms, while lighter colors indicate the least significant terms. **(d)**  
1000 Macrophage localization by immunofluorescence in transversal section from TA muscle at 7 w in

1001 *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mice. Nuclei: DAPI (blue), macrophages (red), plasma membrane  
 1002 (WGA, yellow). Arrowheads point to macrophages. Scale bar = 100 μm. **(e)** Quantification of  
 1003 macrophages. T-test; \*p < 0.05, \*\*p < 0.01. **(f)** Transcriptomic expression changes between diseased and  
 1004 WT mice for the 5 cohorts. The log2foldchange expression of *Anxa2*, *Cilp*, *Fetub*, *Fgfr4*, *Fst*, *Igfbp2*,  
 1005 *Mstn*, *Myl4*, *Runx1*, *S100a4*, *Serpinb1a*, *Sln*, *Sox11*, *Tnnt2* are represented by the bars.

1006  
 1007 **Figure 6.** Common therapy signature in CNM mice treated with different therapies. **(a)** PCA on RNAseq  
 1008 data of the 42 genes commonly rescued in the four cohorts. Each symbol represents a mouse. The three  
 1009 different CNM mouse models are represented by red, orange and yellow colors and the WT controls are  
 1010 represented in green. Treatments are represented by different symbols: squares for the downregulation  
 1011 of *Dnm2*, either by ASO injection (full square) or by genetic cross (boxed +), the triangle represents the  
 1012 overexpression of human *BINI*, the cross represents the administration of tamoxifen. **(b)** Venn diagram  
 1013 illustrating the shared and specific dysregulated genes between rescued and diseased mice in each mouse  
 1014 cohort. **(c)** mRNA expression levels of *Anxa2*, *Cilp*, *Fetub*, *Igfbp2* in CNM mice either diseased or  
 1015 rescued upon therapy, and in untreated and treated controls. Boxplots displaying normalized Ct values.  
 1016 Pairwise significance with p-value < 0.05 calculated by Dunn's Test are represented in bold. **(d)** Among  
 1017 the 42 genes identified in the therapy signature, 35 have human orthologs and 2 (*Scn5a* and *Sbk3*) encode  
 1018 proteins targeted by known drugs.

1019  
 1020 **Figure 7.** Muscle and circulating biomarkers for disease and rescue states. **(a)** Venn diagram illustrating  
 1021 the strategy to extract biomarkers from the comparison of disease and therapy signatures, proteins  
 1022 detected by mass spectrometry in the sera of WT mice, public databases (GTEx, BioGPS, Illumina,  
 1023 GXD), and literature. **(b)** Protein levels of ANXA2, FETUB and CILP in Gastrocnemius with  
 1024 standardization by rouge Ponceau red staining in *Mtm1*<sup>-/-</sup> (Cohort MTM1-a), *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup>  
 1025 mouse models at 7 w. Protein levels are represented as the fold difference from the average of the WT

(4 ≤ n ≤ 9). Student test; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (c) Plasma levels of IGFBP2, ANXA2, CILP and MSTN (ng/mg or pg/mg protein total) from untreated and treated *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup> and *Dnm2*<sup>SL/+</sup> mouse models and WT controls (3 ≤ n ≤ 12). Student test for untreated cohorts; \*\*p < 0.01. Tukey's test for treated cohorts; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

1030

**Table 1:** Description of the mouse cohorts used in the study. Country, age, background, muscle and sequencer used.

**Table 2:** List of the most dysregulated genes in MTM1-CNM mice.

**Table 3:** RNA and protein levels of *Mtm1* (MTM1), *Bin1* (BIN1) and *Dnm2* (DNM2) in *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup>, *Dnm2*<sup>SL/+</sup> and *Dnm2*<sup>RW/+</sup> mice.

1036

**Supplementary information:**

**Table S1.** Number of mice included in the different cohorts for transcriptomic and proteomic experiments.

**Table S2.** List of the 287 genes commonly differentially expressed in MTM1-CNM cohorts (Fig 2a).

**Table S3.** List of the 632 genes differentially expressed in common for MTM1-a, MTM1-b and MTM1-c cohorts.

**Table S4.** List of the specific dysregulated genes expressed in MTM1-CNM cohorts (Fig 2c).

**Table S5.** List of the 68 differentially expressed genes common between mice (Tibialis anterior) and dogs (Vastus Lateralis).

**Table S6.** List of the 53 differentially expressed genes common between mice (Tibialis anterior) and dogs (Biceps Femoris).

**Table S7.** List of DEGs in cohort MTM1-a.

**Table S8.** List of DEP *Mtm1*<sup>-/-</sup> vs WT (Cohort MTM1-a)

**Table S9.** List of the 155 common differentially expressed genes for the MTM1-a, MTM1-b, MTM1-c, DNM2 and BIN1 cohorts.

**Table S10.** List of DEGs in cohort MTM1-b.

**Table S11.** List of DEGs in cohort MTM1-c.

**Table S12.** List of DEGs in cohort DNM2.

**Table S13.** List of DEGs in cohort BIN1.

**Table S14.** List of the specific dysregulated genes expressed in MTM1, DNM2 and BIN1 cohorts (Fig 5b).

**Table S15.** List of the 42 common differentially expressed genes for the MTM1-a, MTM1-b, DNM2 and BIN1 cohorts.

**Table S16.** List of proteins retrieved by mass spectrometry in serum of WT mice at 8 w.

**Table S17.** List of primers used for RT-qPCR.

**Figure S1. (a)** Venn diagram illustrating the common dysregulated genes based on the MTM1 vs controls comparison in three different species: human, mice and dog (Biceps femoris). **(b-c)** Gene Ontology (GO) enrichment analysis of the specific differentially expressed genes in **(b)** Human cohort and **(c)** in Dog cohort. The 20 GO biological process terms with the lowest p-value are displayed.

**Figure S2.** PCA related to Fig 5b. **(a)** PC1 and PC2 and **(b)** PC1 and PC3 are represented.

**Figure S3.** GO enrichment analysis for biological processes (BP), cellular component (CC) and molecular function (MF) of the differentially expressed genes in *Mtm1*<sup>-/-</sup> vs WT mice (N =1175 genes) at **(a)** 2 w and **(b)** at 7 w (N=1981 genes). GO terms with highest ratio and lowest q-value are represented. The ratio represents the number of genes dysregulated divided by the total number of genes in the

category. The color scale is based on the q-value, dark colors indicate most significantly over-represented terms, while lighter colors indicate the least significant terms.

**Figure S4.** mRNA expression levels of genes of interest in diseased, rescued and control mice in the MTM1-a cohort at 2 w. Boxplots displaying normalized Ct values. Pairwise significance calculated by Dunn's test,  $p < 0.05$  are represented in bold.

**Figure S5.** mRNA expression levels of genes of interest in diseased, rescued and control mice in the MTM1-a cohort at 7 w. Boxplots displaying normalized Ct values. Pairwise significance calculated by Dunn's test,  $p < 0.05$  are represented in bold.

**Figure S6.** GO enrichment analysis for biological processes (BP), cellular component (CC) and molecular function (MF) of the differentially expressed proteins in *Mtm1*<sup>-/-</sup> vs WT mice (N =168 proteins) at **(a)** 2w and **(b)** at 7 w (N=496 proteins). GO terms with highest ratio and lowest q-value are represented. The ratio represents the number of proteins dysregulated divided by the total number of proteins in the category. The color scale is based on the q-value, dark colors indicate most significantly over-represented terms, while lighter colors indicate the least significant terms.

**Figure S7.** Pearson correlation between mRNA and protein levels measured by RNASeq and mass spectrometry **(a)** at 2 w and **(b)** at 7 w.

**Figure S8.** MTM1 and BIN1 protein levels in *Dnm2*<sup>SL/+</sup> Tibialis anterior muscles. MTM1, BIN1 and DNM2 protein levels in *Dnm2*<sup>RW/+</sup> TA muscles. MTM1 protein level in *Bin1*<sup>mck-/-</sup> TA muscles. Pairwise significance calculated by t-test,  $p < 0.05$  are represented in bold.

1099 **Figure S9.** Number of dysregulated genes and the associated PCA for each cohort for four comparisons  
1100 (Disease vs WT, Disease vs Rescue, Rescue vs Disease, and WT treated vs WT). **(a)** Cohort MTM1-b.  
1101 **(b)** Cohort MTM1-c. **(c)** Cohort DNM2. **(d)** Cohort BIN1.

1102  
1103 **Figure S10.** mRNA expression levels of genes of interest in diseased, rescued and control mice in the  
1104 DNM2 cohort at 7 w. Boxplots displaying normalized Ct values. Pairwise significance calculated by  
1105 Dunn's test,  $p < 0.05$  are represented in bold.

1106  
1107 **Figure S11.** mRNA expression levels of genes of interest in diseased, rescued and control mice in the  
1108 MTM1-b cohort at 7 w. Boxplots displaying normalized Ct values. Pairwise significance calculated by  
1109 Dunn's test,  $p < 0.05$  are represented in bold.

1110  
1111 **Figure S12.** mRNA expression levels of genes of interest in diseased, rescued and control mice in the  
1112 BIN1 cohort at 7 w. Boxplots displaying normalized Ct values. Pairwise significance calculated by  
1113 Dunn's test,  $p < 0.05$  are represented in bold.

1114  
1115 **Figure S13.** Gene Ontology (GO) enrichment analysis of the specific differentially expressed genes **(a)**  
1116 in MTM1 cohort **(b)** in BIN1 cohort and **(c)** in DNM2 cohort. The 20 GO biological process terms with  
1117 the lowest p-value are displayed.

1118  
1119 **Figure S14.** Blot related to Fig 7b.

1120  
1121 **Figure S15. (a)** Histogram displaying the proportion of genes according to their status and the metric per  
1122 cohort. **(b)** Barplot showing the number of genes dysregulated and the status of these genes through the  
1123 different therapies.

1124

1125

1126

1127 **Table1:** Description of the mouse cohorts used in the study. Country, age, background,  
1128 muscle and sequencer used.

	Country	Age	Background	Muscle	Sequencer
Cohort MTM1-a	France	7w	129Pas	Tibialis anterior	Hiseq4000
Cohort 2 MTM1-b	France	7w	50% 129Pas; 50% C57BL/6N	Tibialis anterior	Hiseq4000
Cohort 3 MTM1-c	Switzerland	7w	129Pas	Tibialis anterior	Hiseq4000
Cohort 4 MTM1-d	Canada (Maani et al., 2018)	5w	C57BL/6J	Quadriceps	Hiseq2500
Cohort DNM2	France	7w	C57BL/6N	Tibialis anterior	Hiseq4000
Cohort BIN1	France	7w	C57BL/6N	Tibialis anterior	Hiseq4000

1129

1130 **Table2:** List of the most dysregulated genes in MTM1-CNM mice.

Gene name	Log2FC Cohort MTM1- a	Log2FC Cohort MTM1-b	Log2FC Cohort MTM1-c	Log2FC Cohort MTM1-d
Sox11	6,16	4,70	5,80	3,97
Krt18	5,25	3,20	7,78	5,23
Gm28653	5,24	2,64	5,13	3,80
Mt3	4,76	3,31	5,28	4,66
Gm13583	4,65	3,17	6,53	8,15
Msln	4,61	3,28	3,96	4,19
Hsf2bp	4,32	3,48	4,24	4,21
Fosl1	4,21	2,69	4,81	6,85
C130080G10Rik	-1,88	-2,13	-3,36	-5,96
Amd1	-2,04	-2,66	-3,17	-2,72
Nt5c1a	-2,05	-2,54	-2,79	-3,77
Fam19a4	-2,31	-3,00	-2,55	-3,77
Ighm	-2,33	-2,89	-3,34	-2,55
Mtm1	-2,43	-2,12	-2,10	-2,55
Edn3	-2,51	-3,27	-2,93	-1,98
Cdh4	-2,57	-3,91	-3,63	-2,85
Mstn	-2,59	-1,79	-1,89	-2,72

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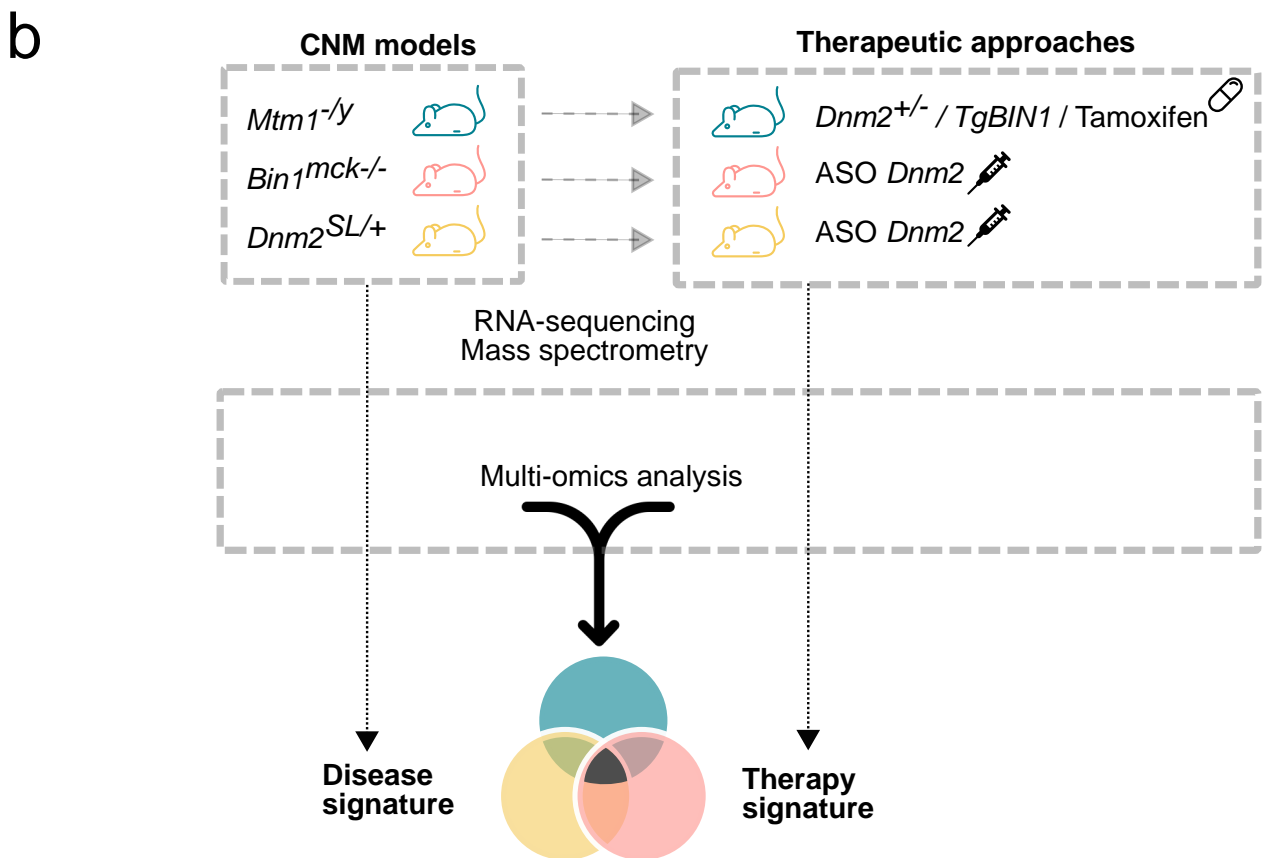
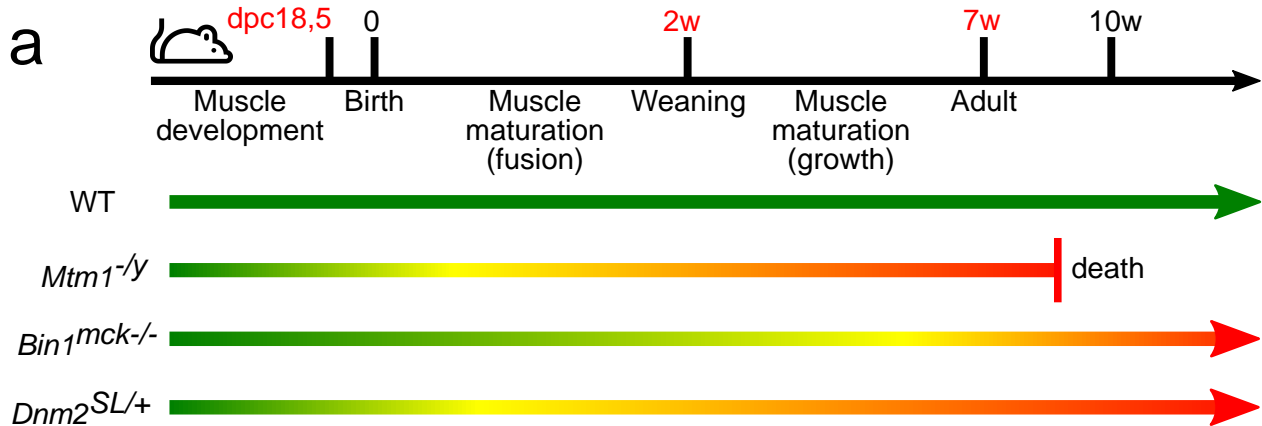


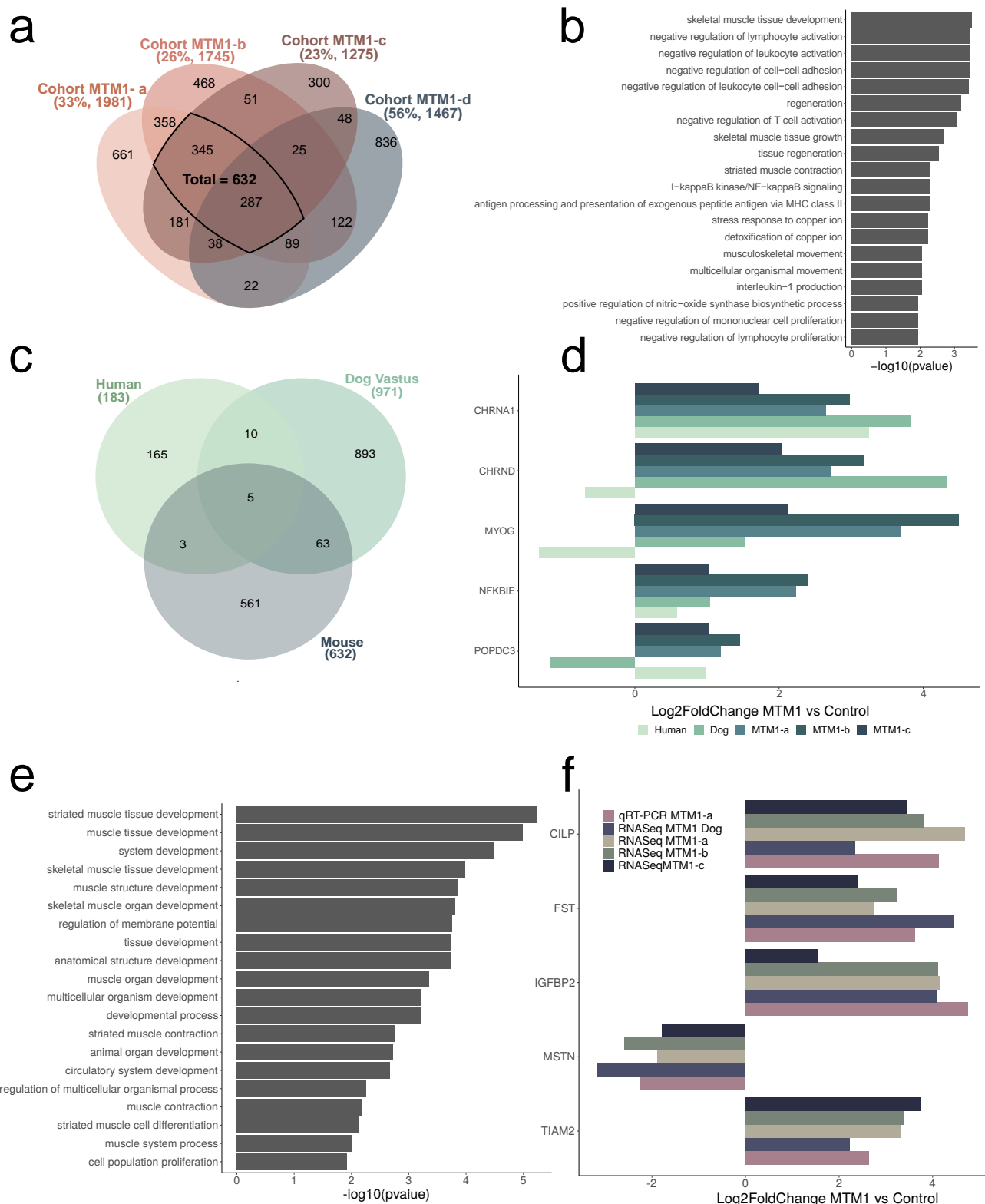
**Table3:** RNA and protein levels of Mtm1 (MTM1), Bin1 (BIN1) and Dnm2 (DNM2) in *Mtm1*<sup>-/-</sup>, *Bin1*<sup>mck-/-</sup>, *Dnm2*<sup>SL/+</sup> and *Dnm2*<sup>RW/+</sup> mice.

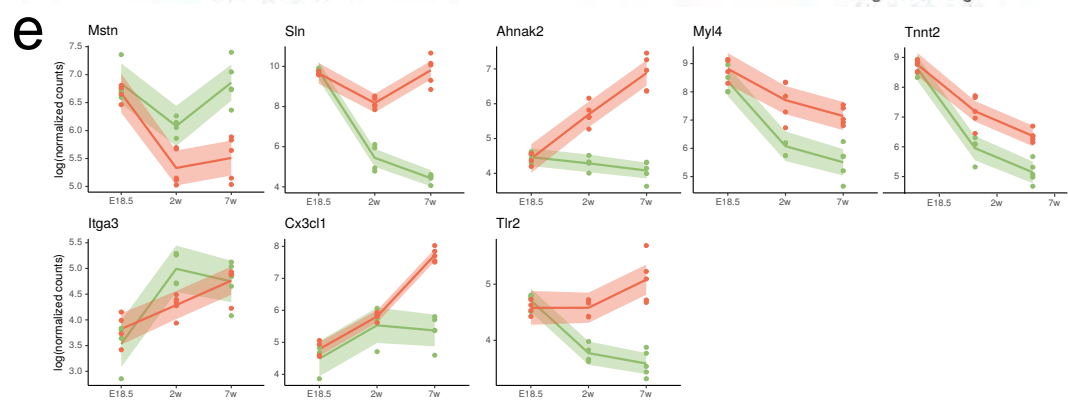
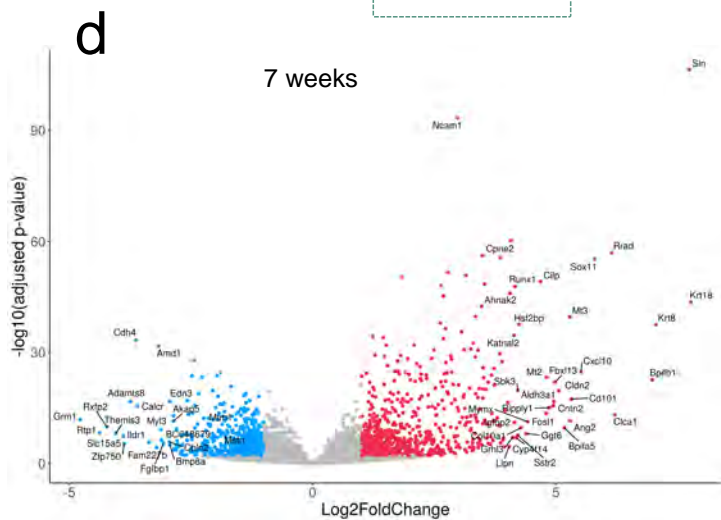
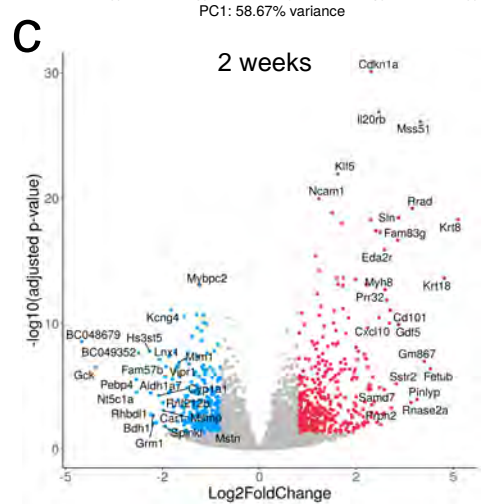
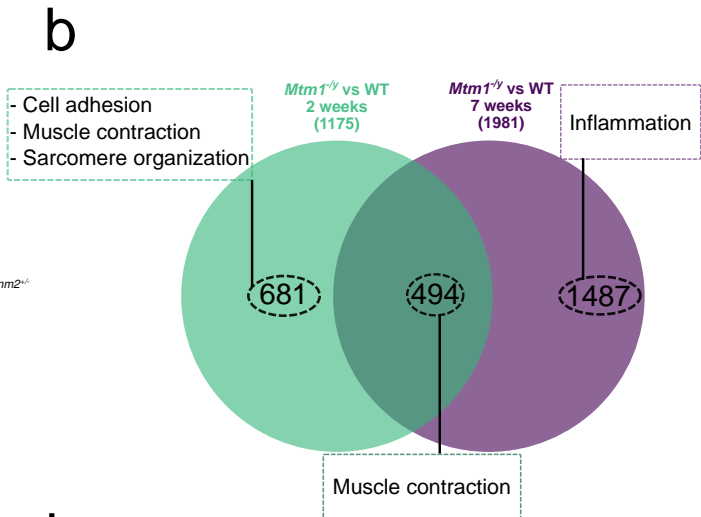
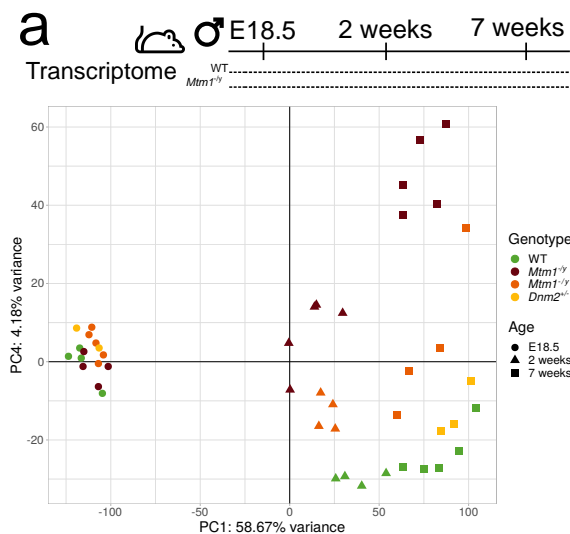
Mouse line	RNA/Protein	MTM1	BIN1	DNM2
<b><i>Mtm1</i><sup>-/-</sup></b>	Protein (WB)	Absent Age 5w (Cowling et al. 2014)	Fold = 2 P < 0.05 Age 7w (Lionello et al., 2019)	Fold = 2.5 P < 0.001 Age 5w (Cowling et al. 2014)
	RNA (qPCR)	Fold = 0.5 P = 0.0095 Age 7w	n.d.	Fold = 1.3 P < 0.05 Age 7w
	RNA (transcriptomic)	Fold = 0.2 P = 1.98E-09 Age 7w	Fold = 1.4 P = 0.046 Age 7w	Fold = 1.02 P = 0.95 Age 7w
<b><i>Bin1</i><sup>mck-/-</sup></b>	Protein (WB)	Fold = 1.07 P = 0.95 Age 8w	Absent Unpublished	Fold = 1.5 P = 0.052 Age 8w Unpublished
	RNA (qPCR)	Fold = 0.97 P = 0.28 Age 7w	Absent P = 0.0091 Age 7w	Fold = 1.4 P < 0.05 Age 8w Unpublished
	RNA (transcriptomic)	Fold = 0.93 P = 0.63 Age 7w	Fold = 0.10 P = 1.63E-209 Age 7w	Fold = 1.1 P = 0.42 Age 7w
<b><i>Dnm2</i><sup>SL/+</sup></b>	Protein (WB)	Fold = 0.7 Pval = 0.016 Age 8w	Fold = 0.95 Pval > 0.05 Age 8w	Fold = 2.2 Pval = 0.057 Age 8w
	RNA (qPCR)	Fold = P = 0.15 Age 7w	n.d.	Fold = P = 0.014 Age 7w
	RNA (transcriptomic)	Fold = 0.87 P = 0.023 Age 7w	Fold = 1.37 P = 2.34E-09 Age 7w	Fold = 1.04 P = 0.77 Age 7w

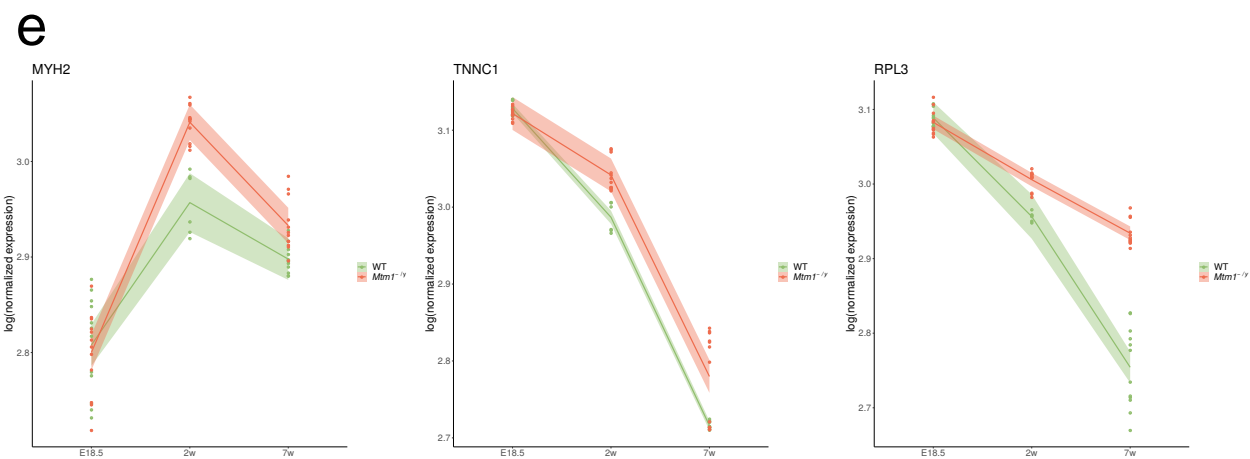
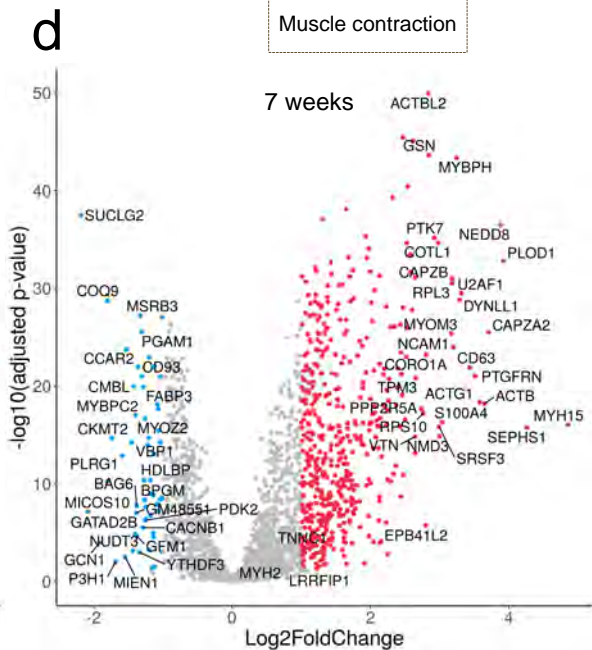
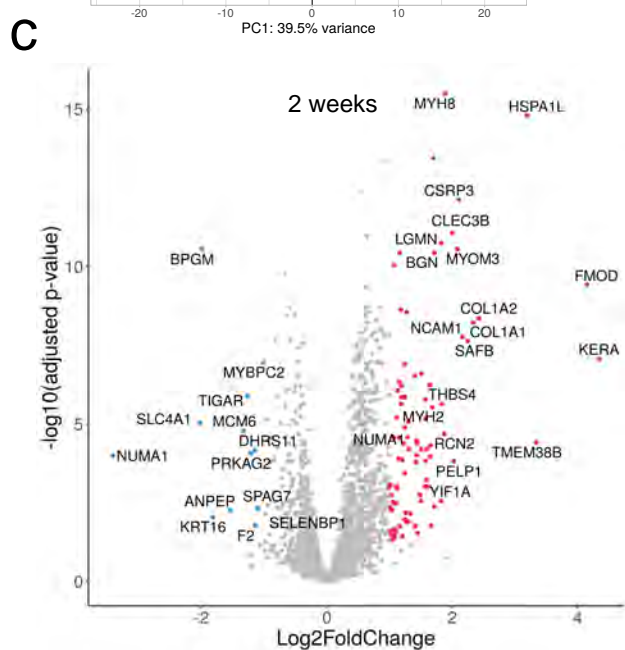
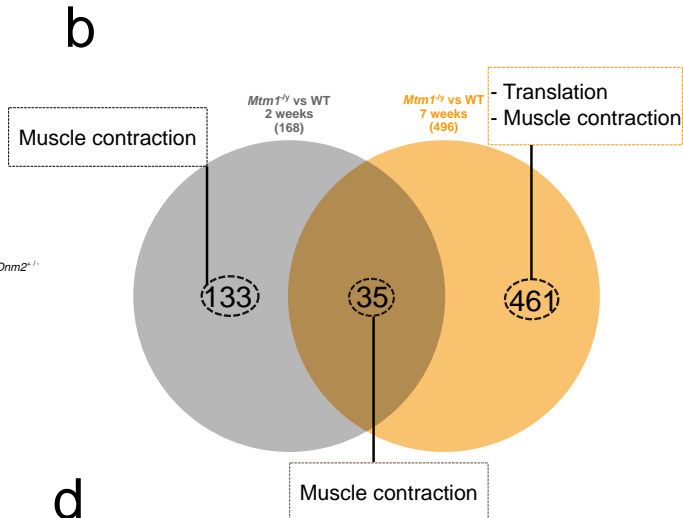
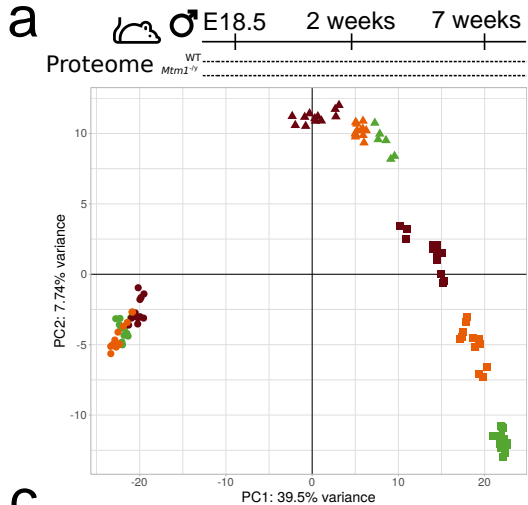
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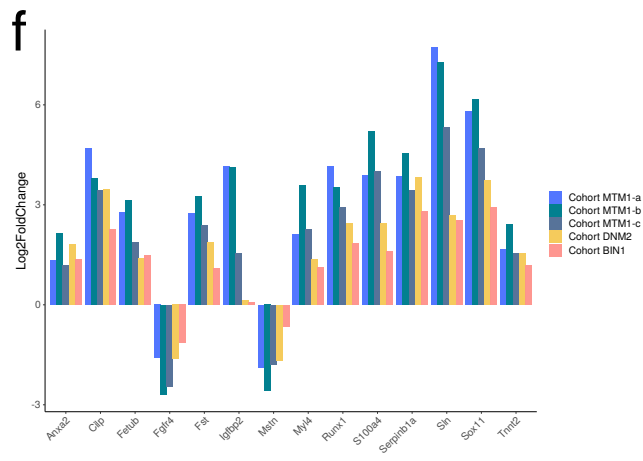
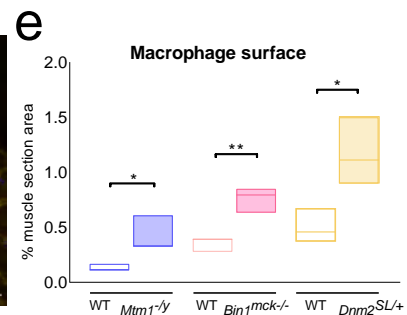
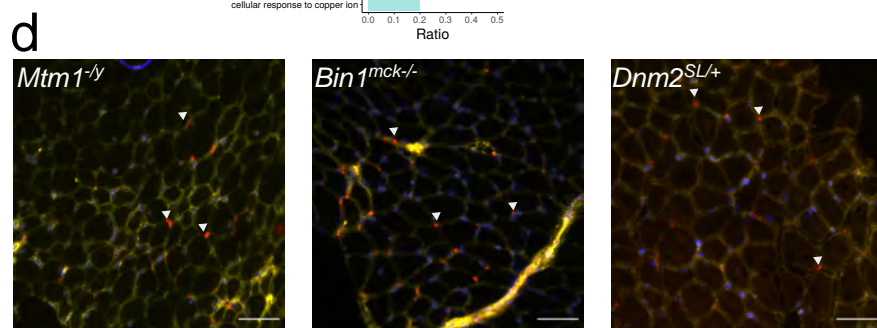
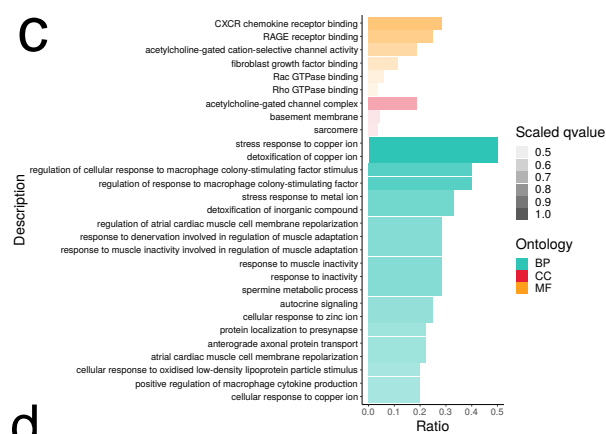
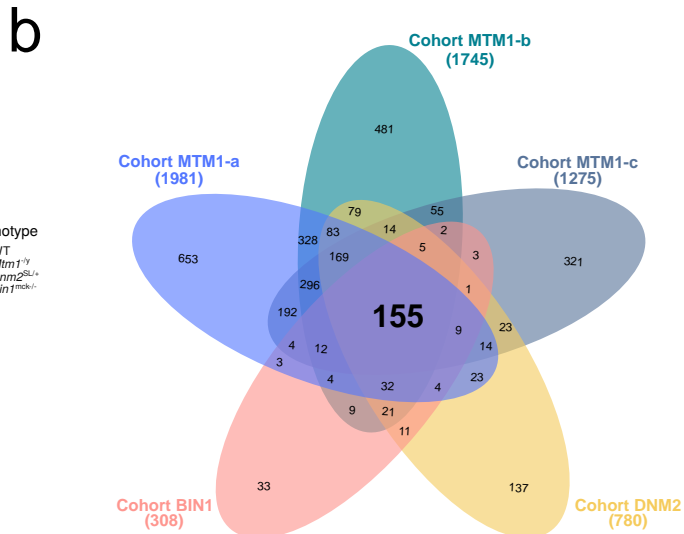
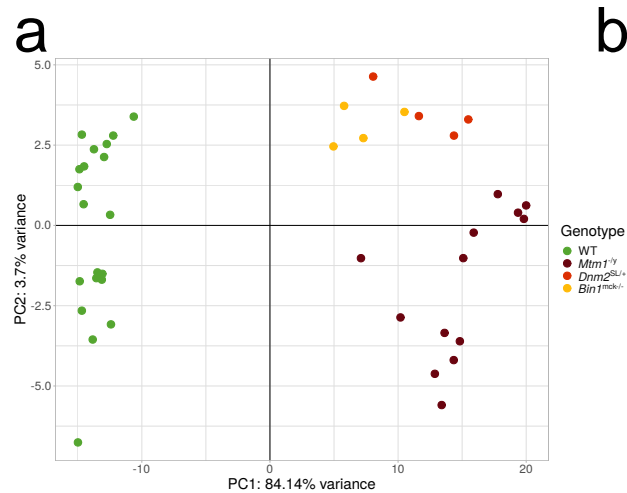
<i>Dnm2</i> <sup>RW/+</sup>	Prot	Fold = 1.2 P = 0.4 Age 7w	Fold = 1.8 P = 0.07 Age 7w	Fold = 1.05 P = 0.6 Age 7w
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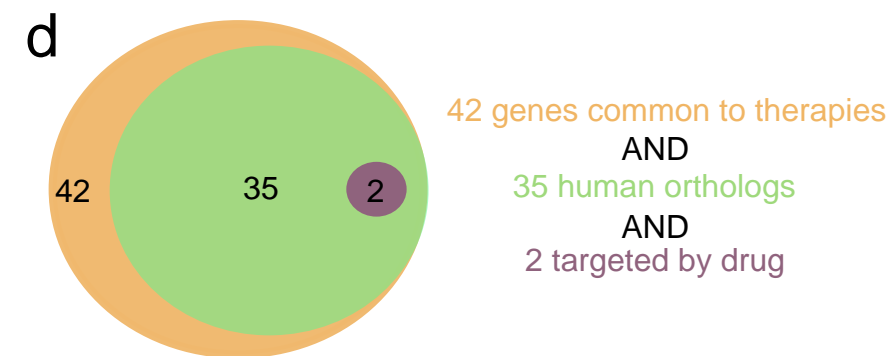
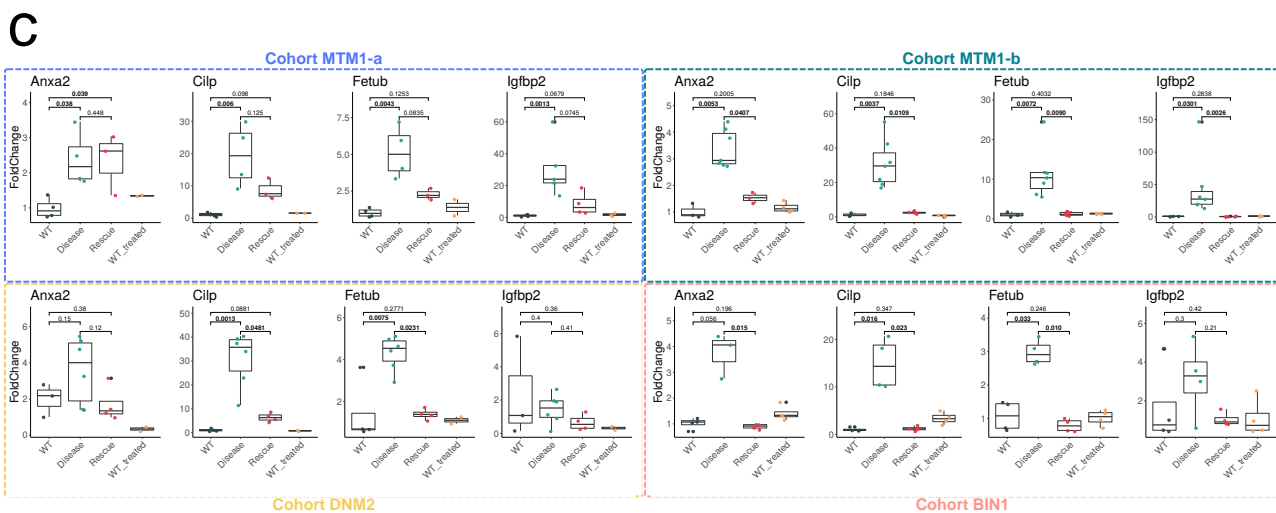
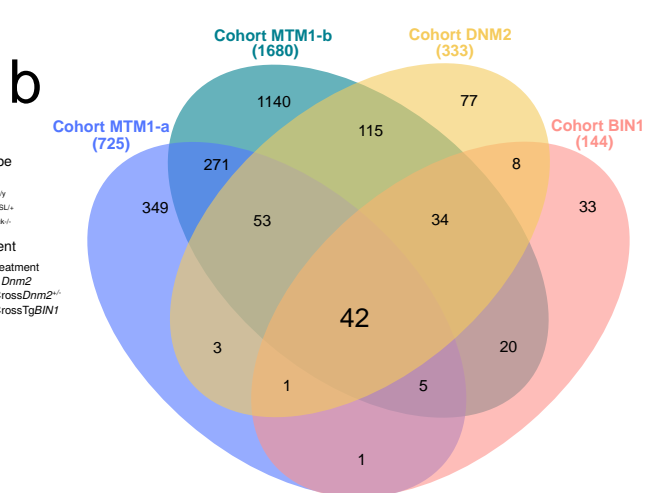
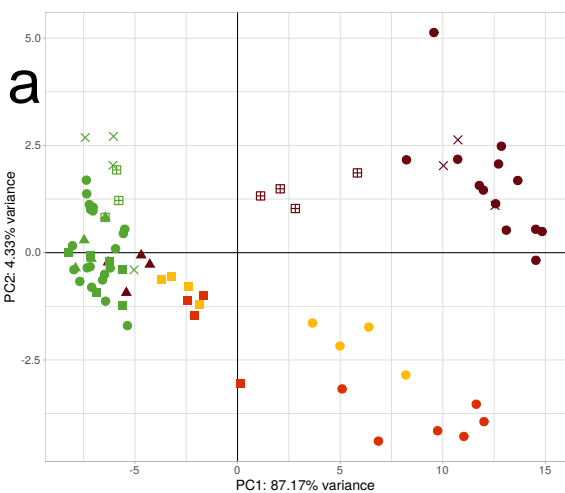




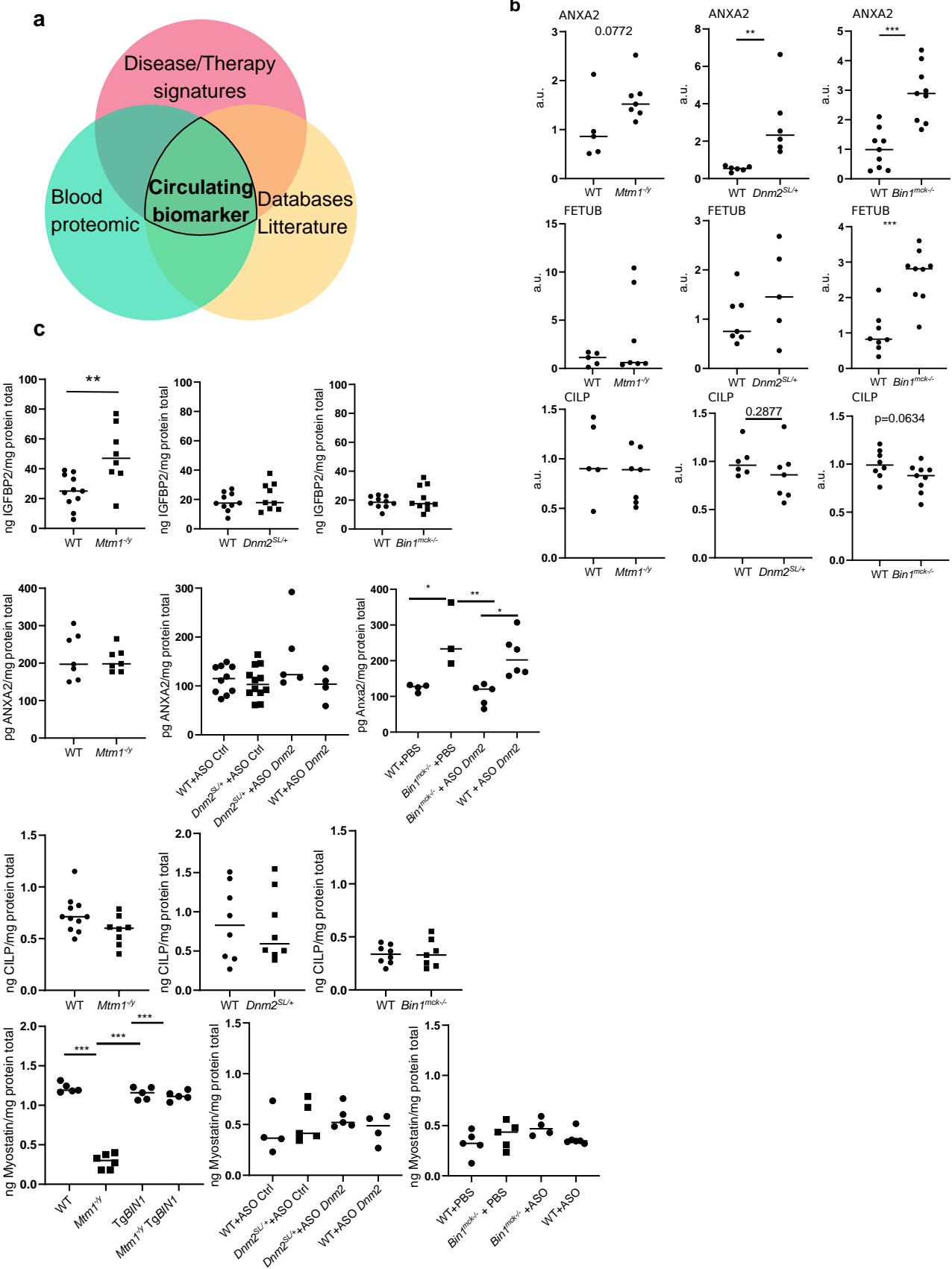




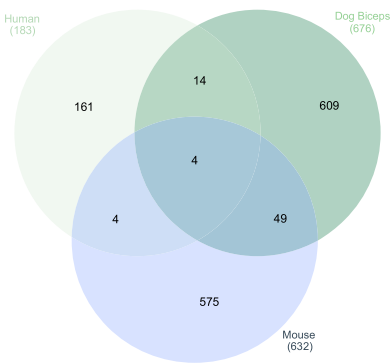




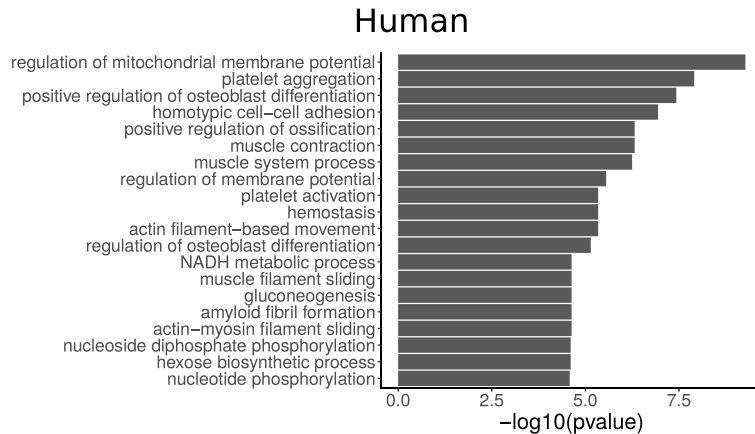




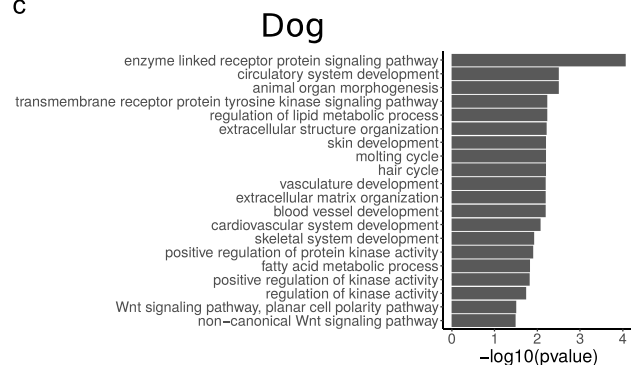
a



b

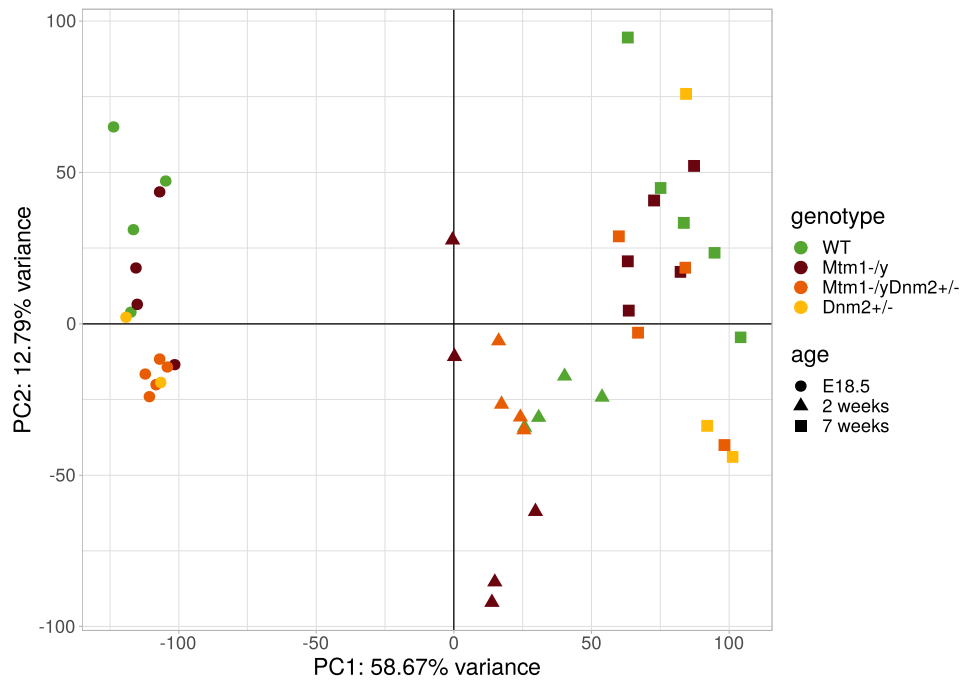


c

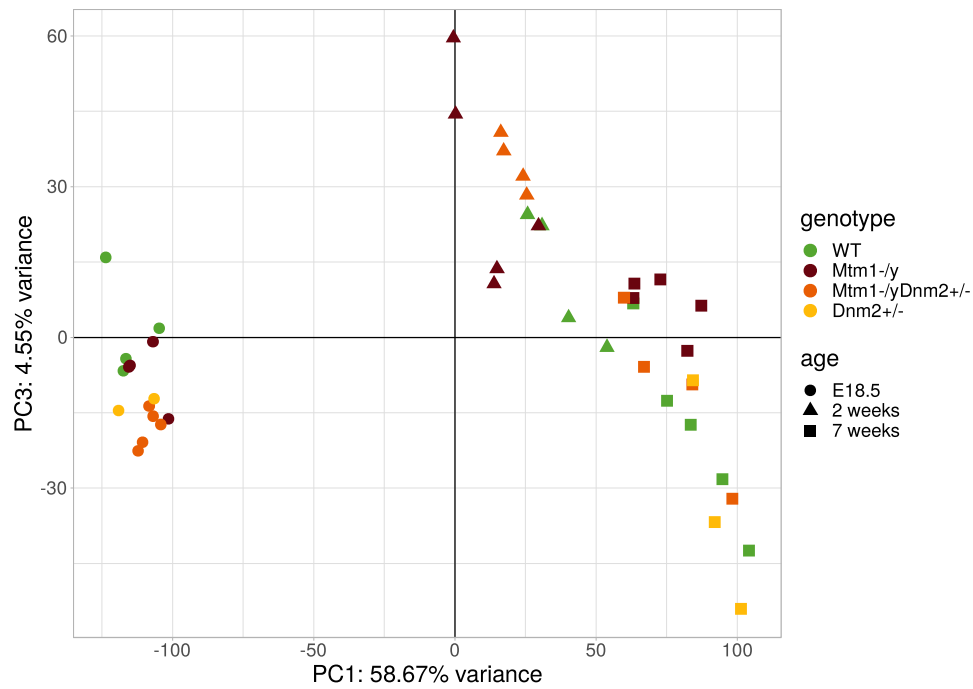


Supplementary Figure S2

a

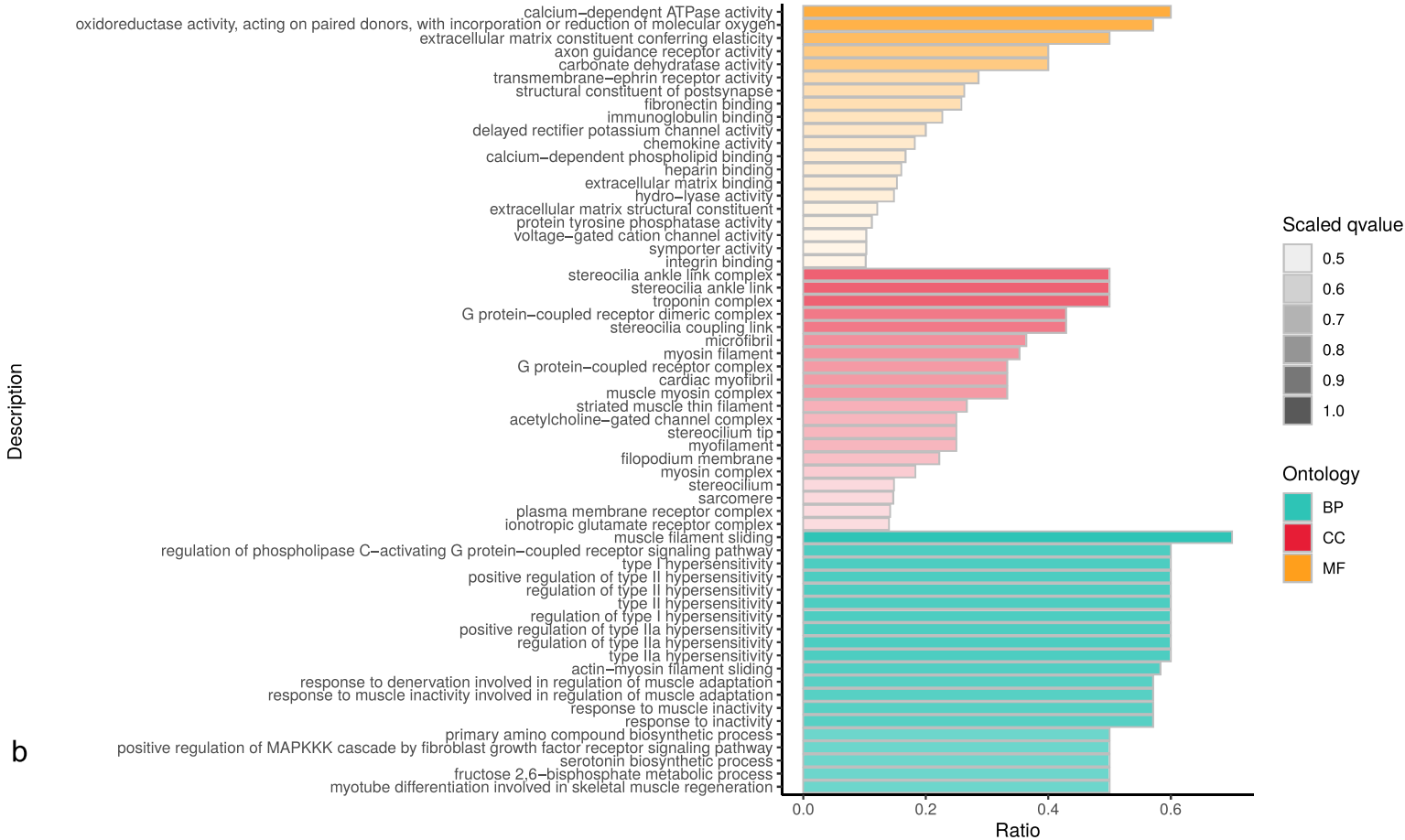


b

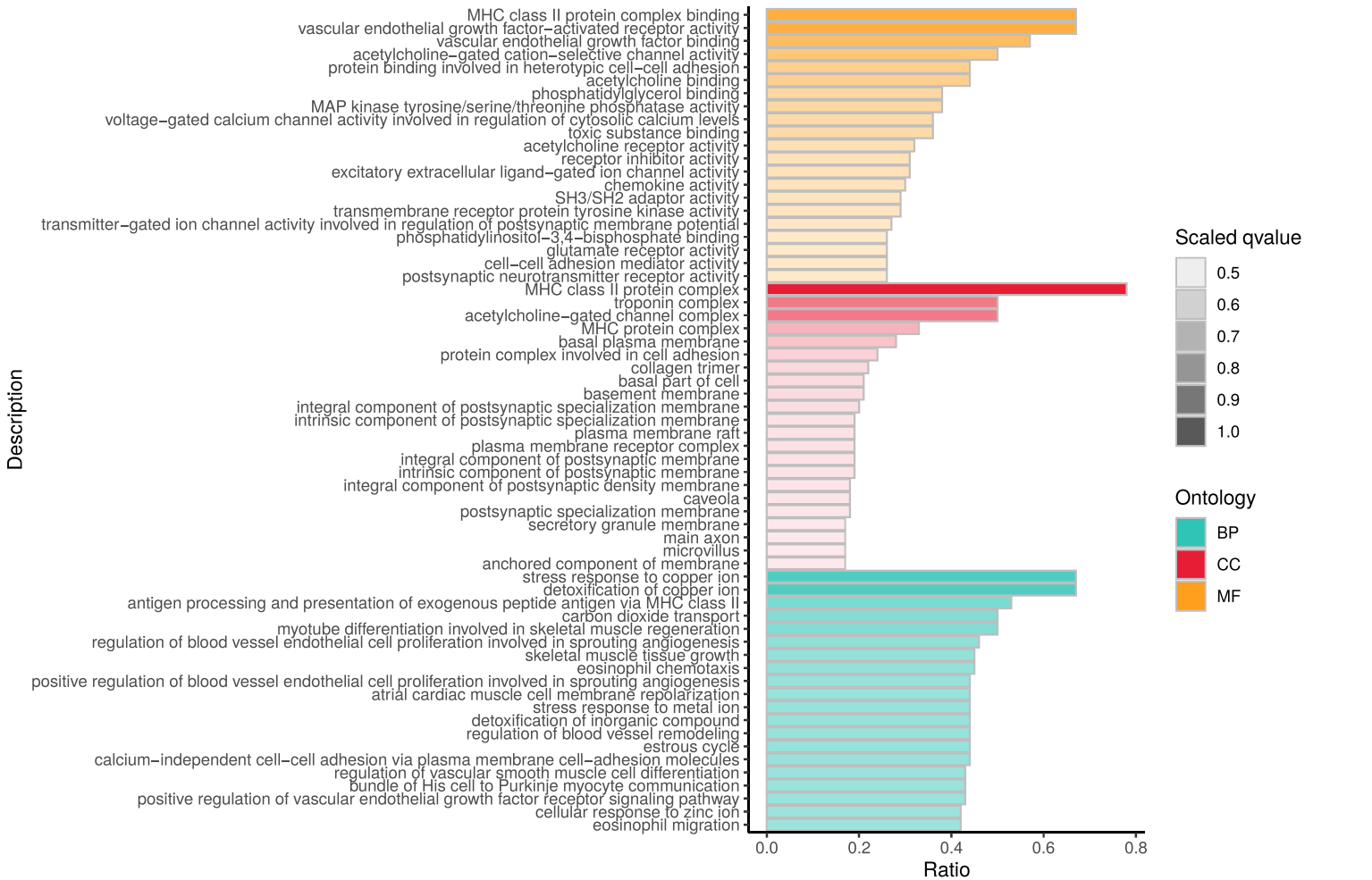


Supplementary Figure S3

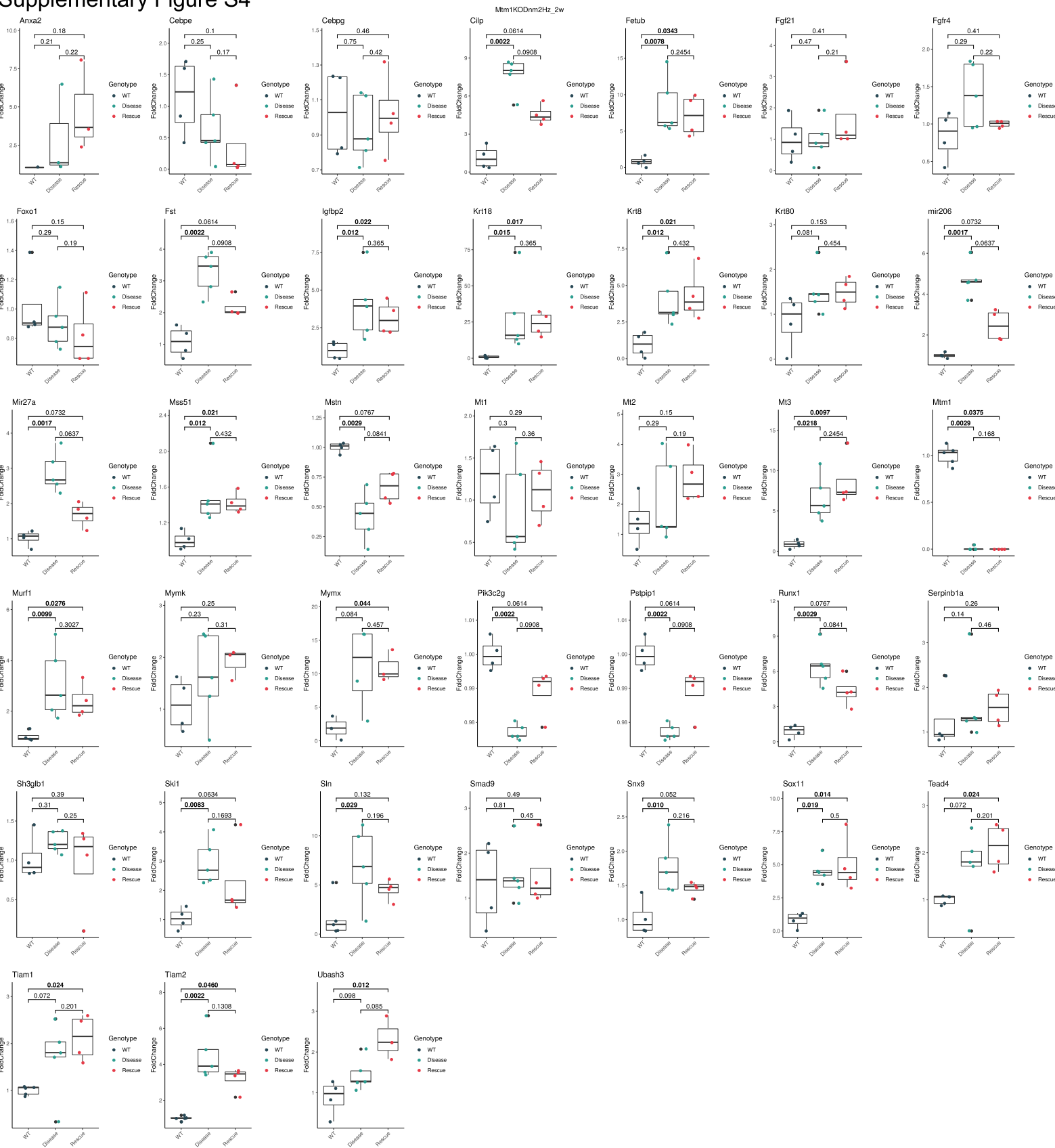
a



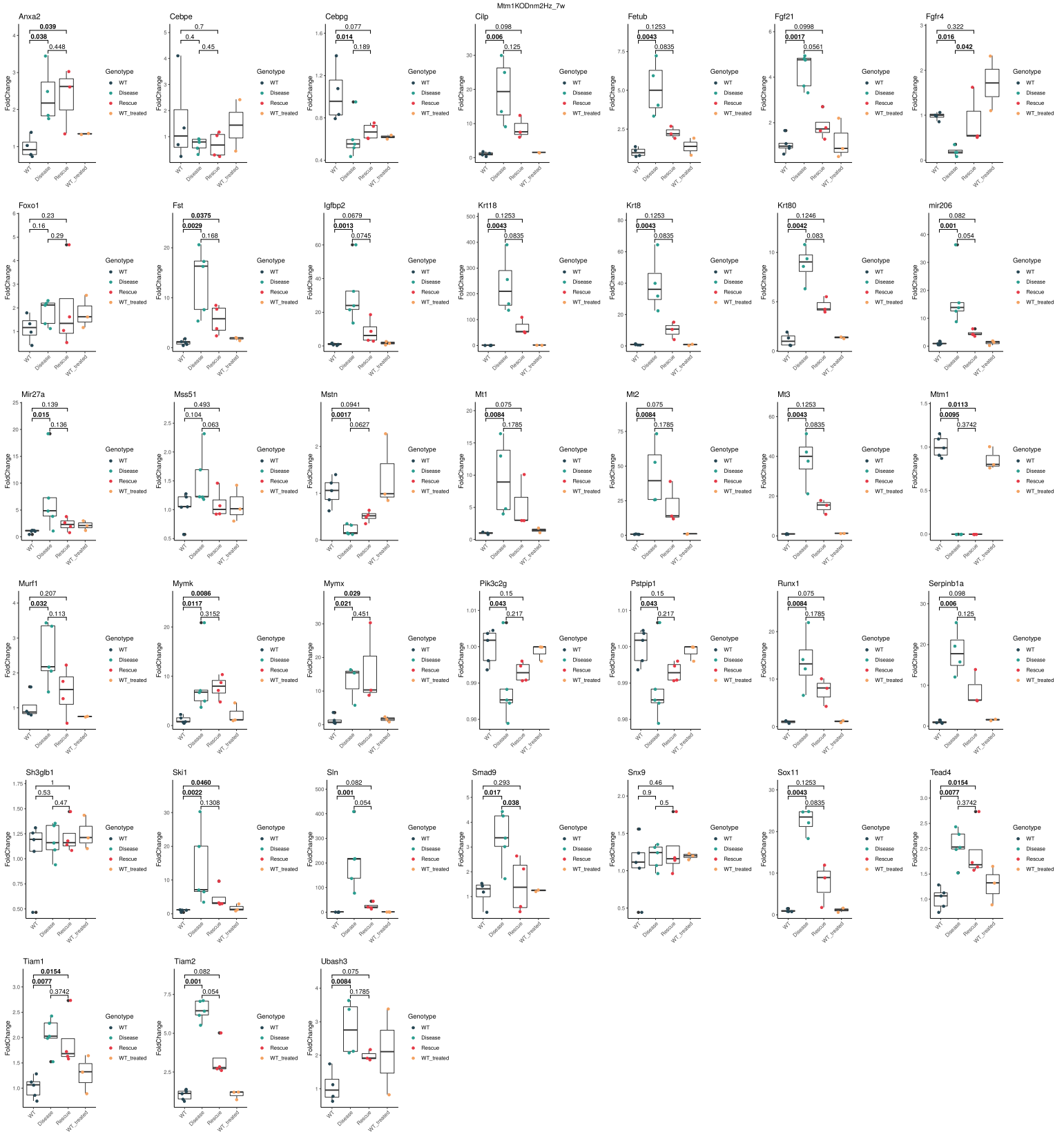
b



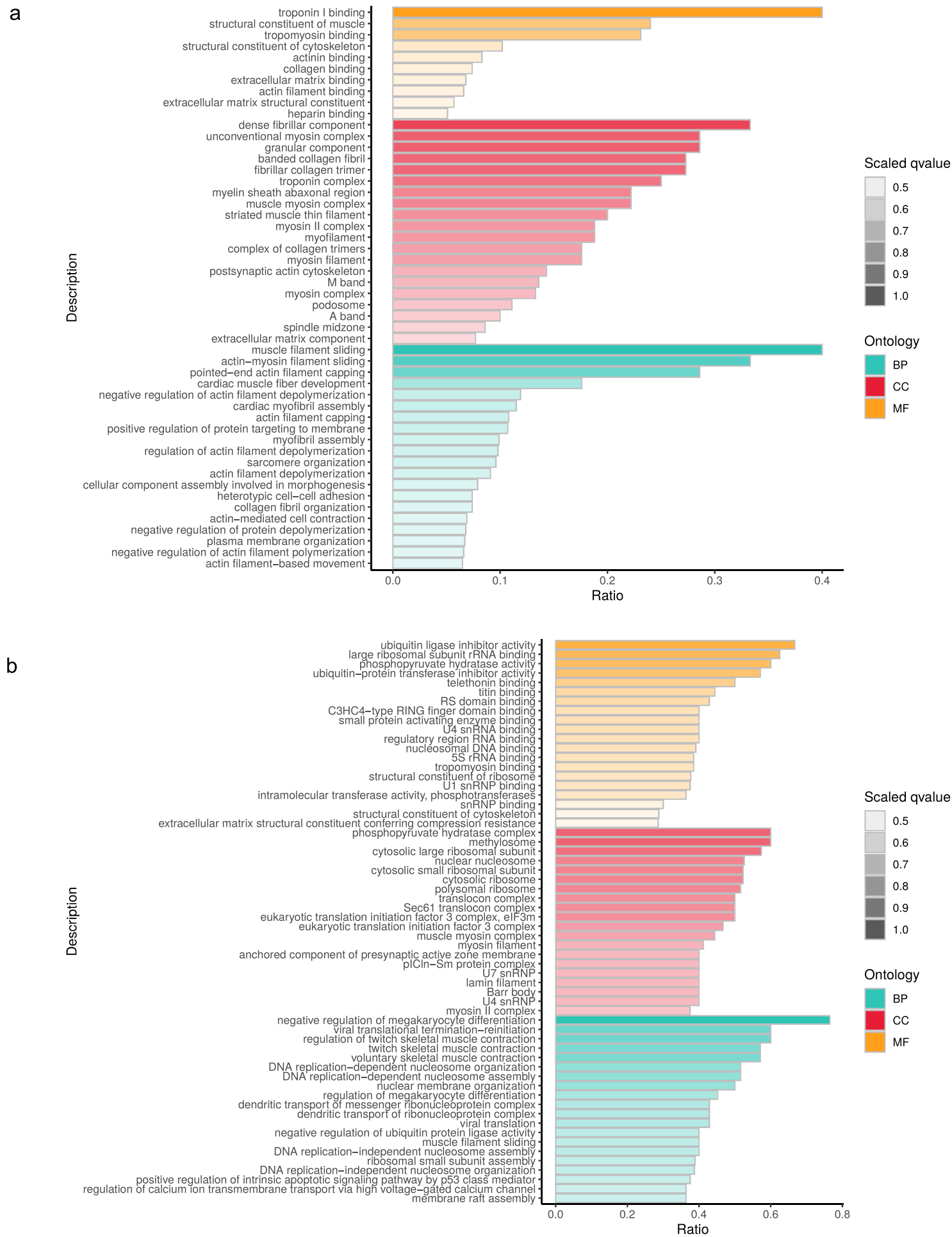
Supplementary Figure S4



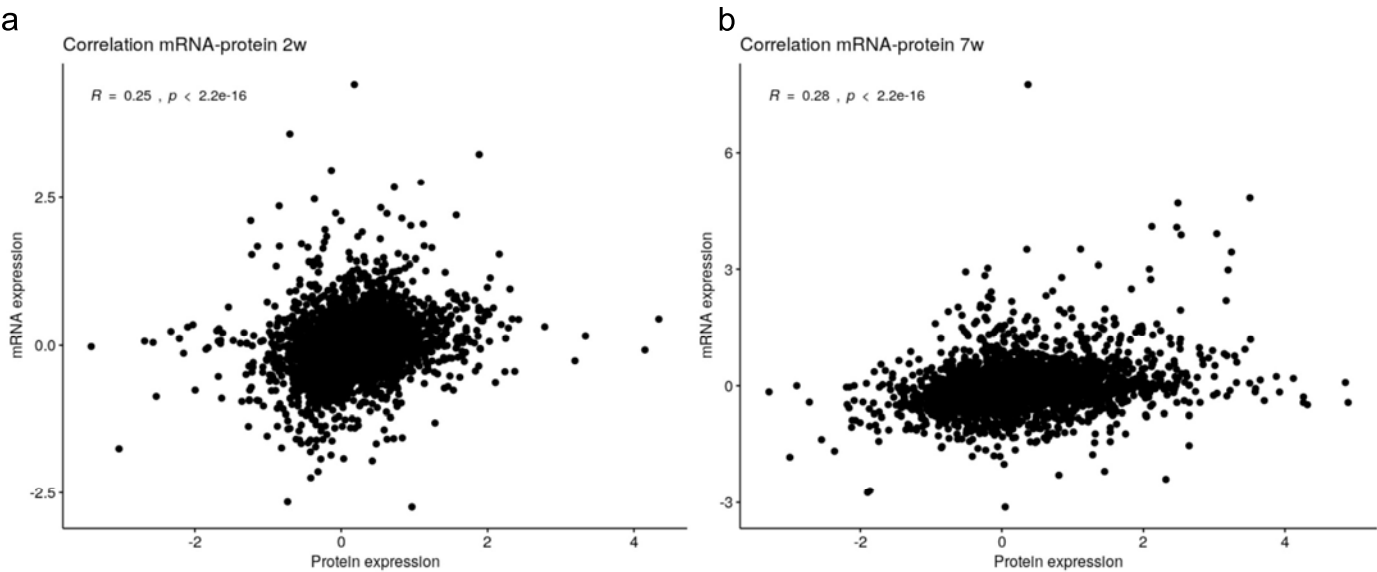
## Supplementary Figure S5



Supplementary Figure S6



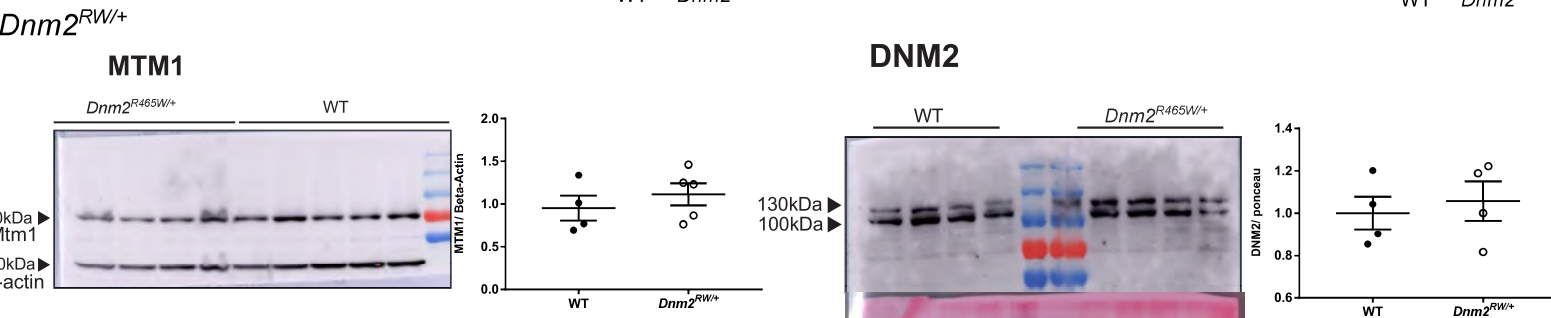
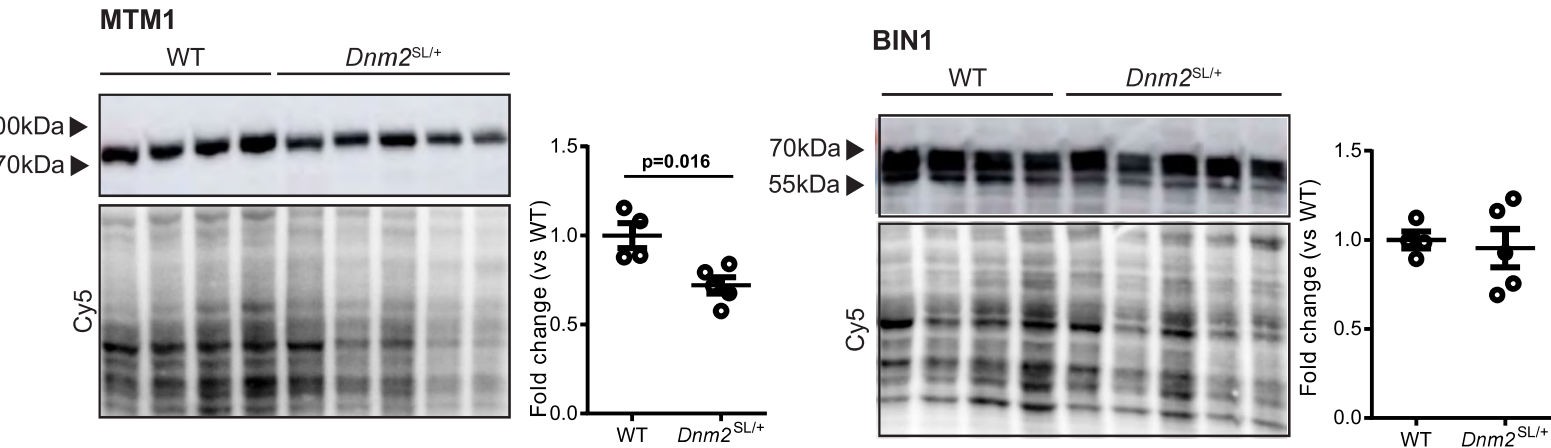
Supplementary Figure S7



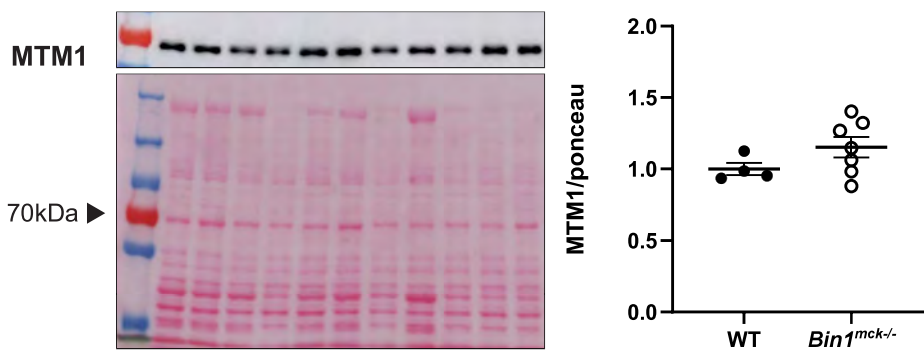


Supplementary Figure S8

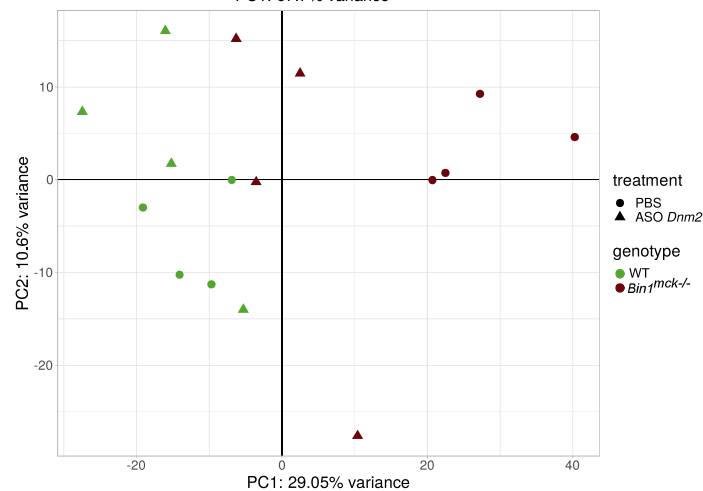
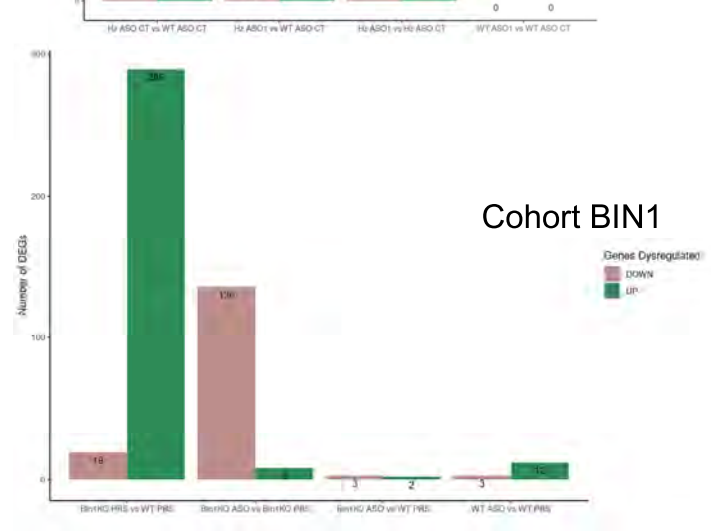
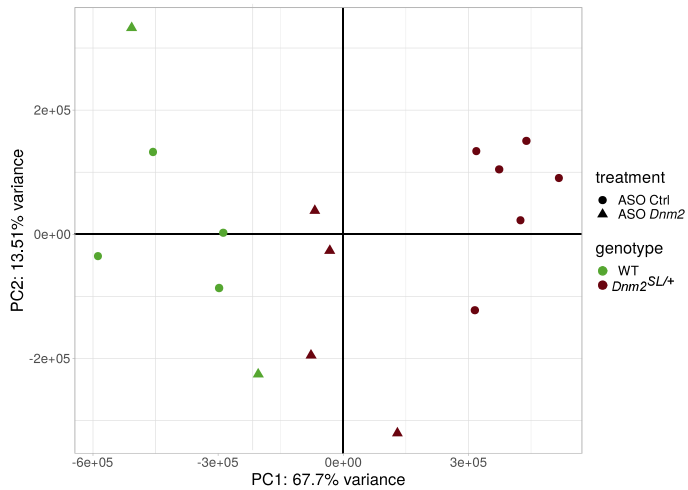
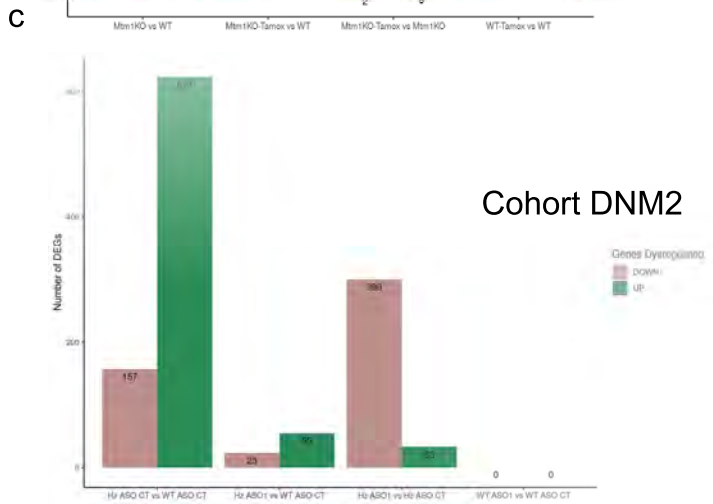
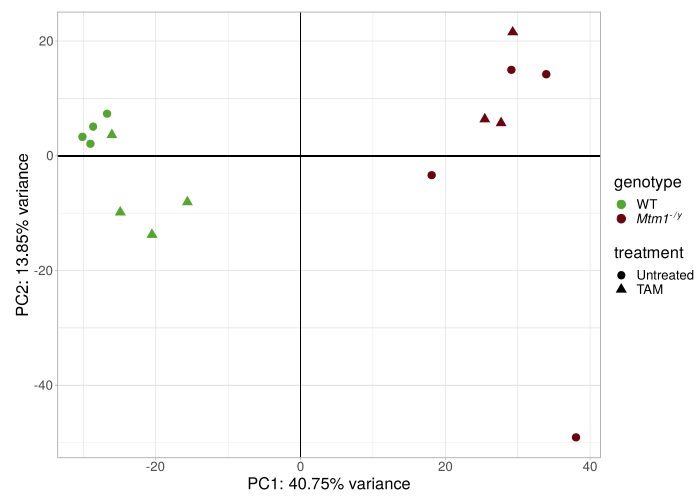
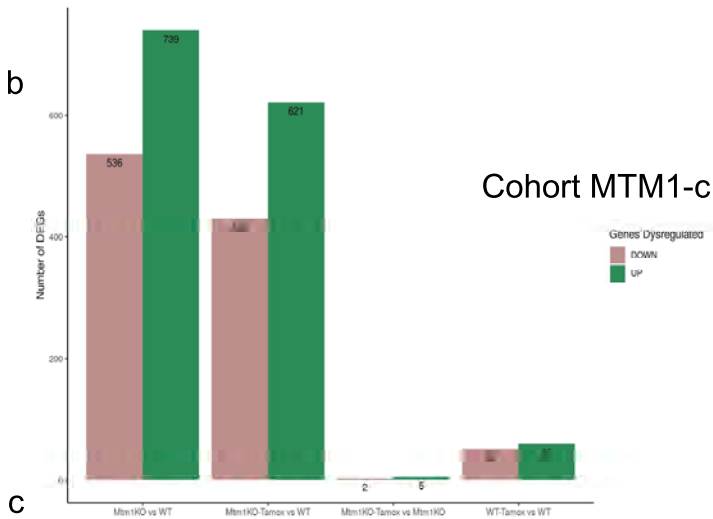
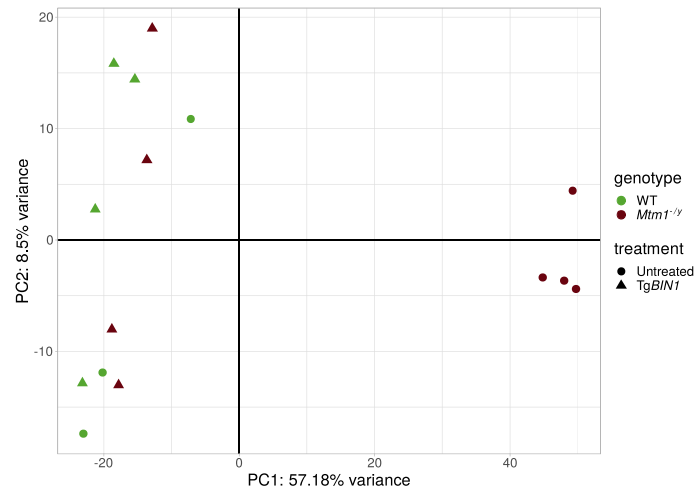
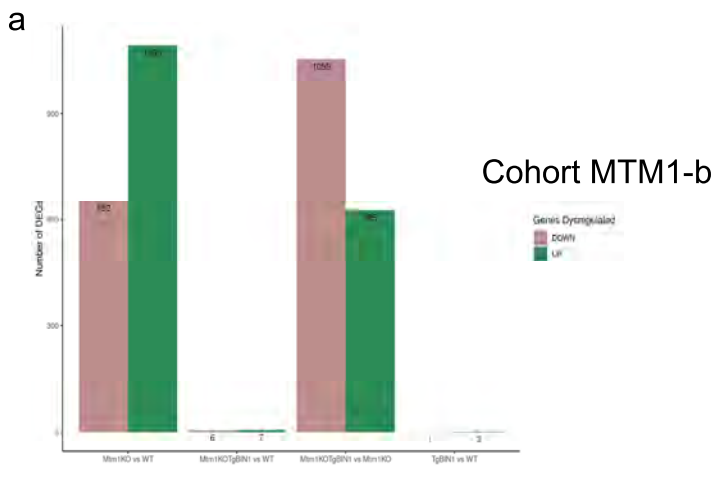
*Dnm2*<sup>SL/+</sup>



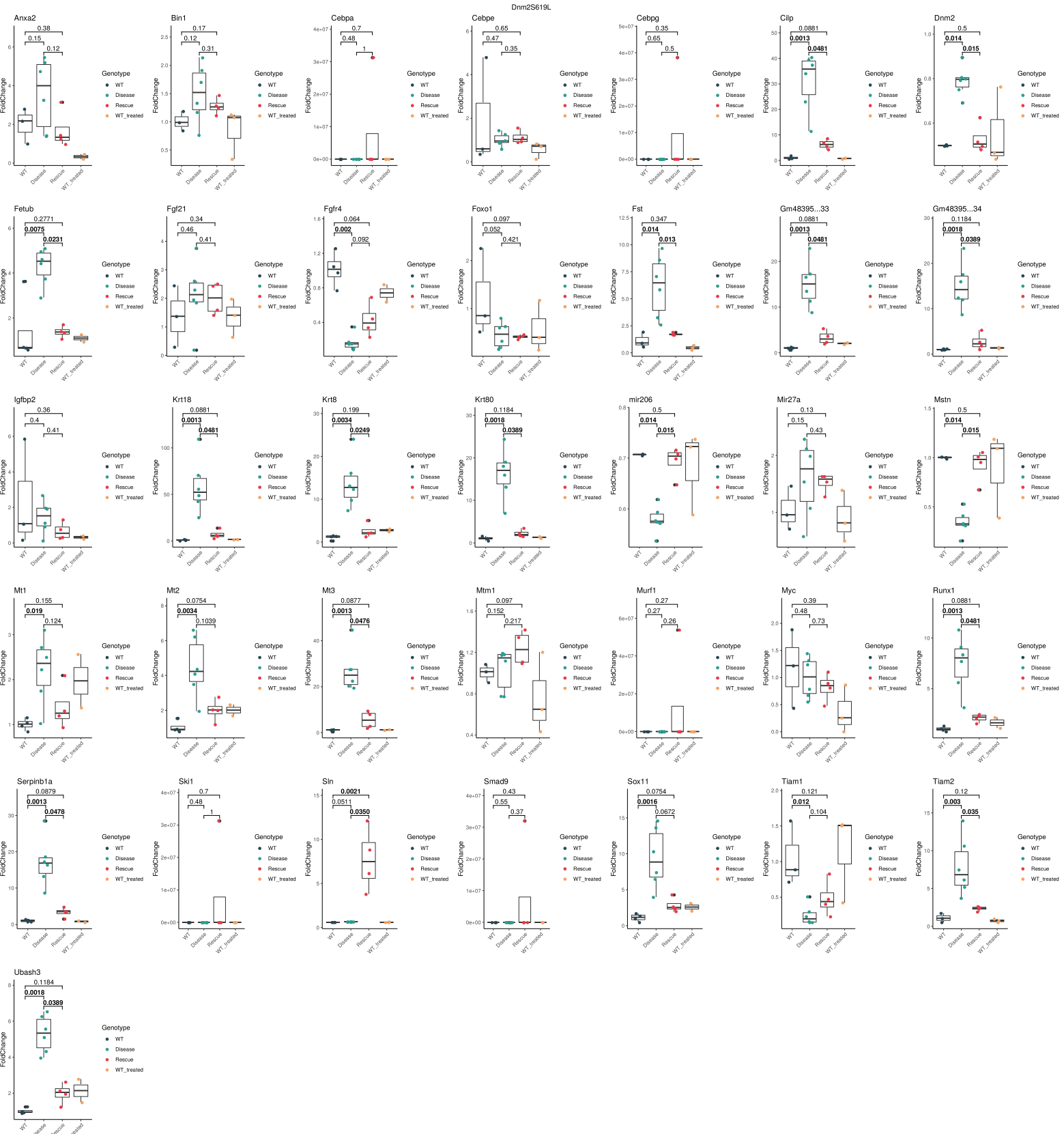
*Bin1*<sup>mck-/-</sup>



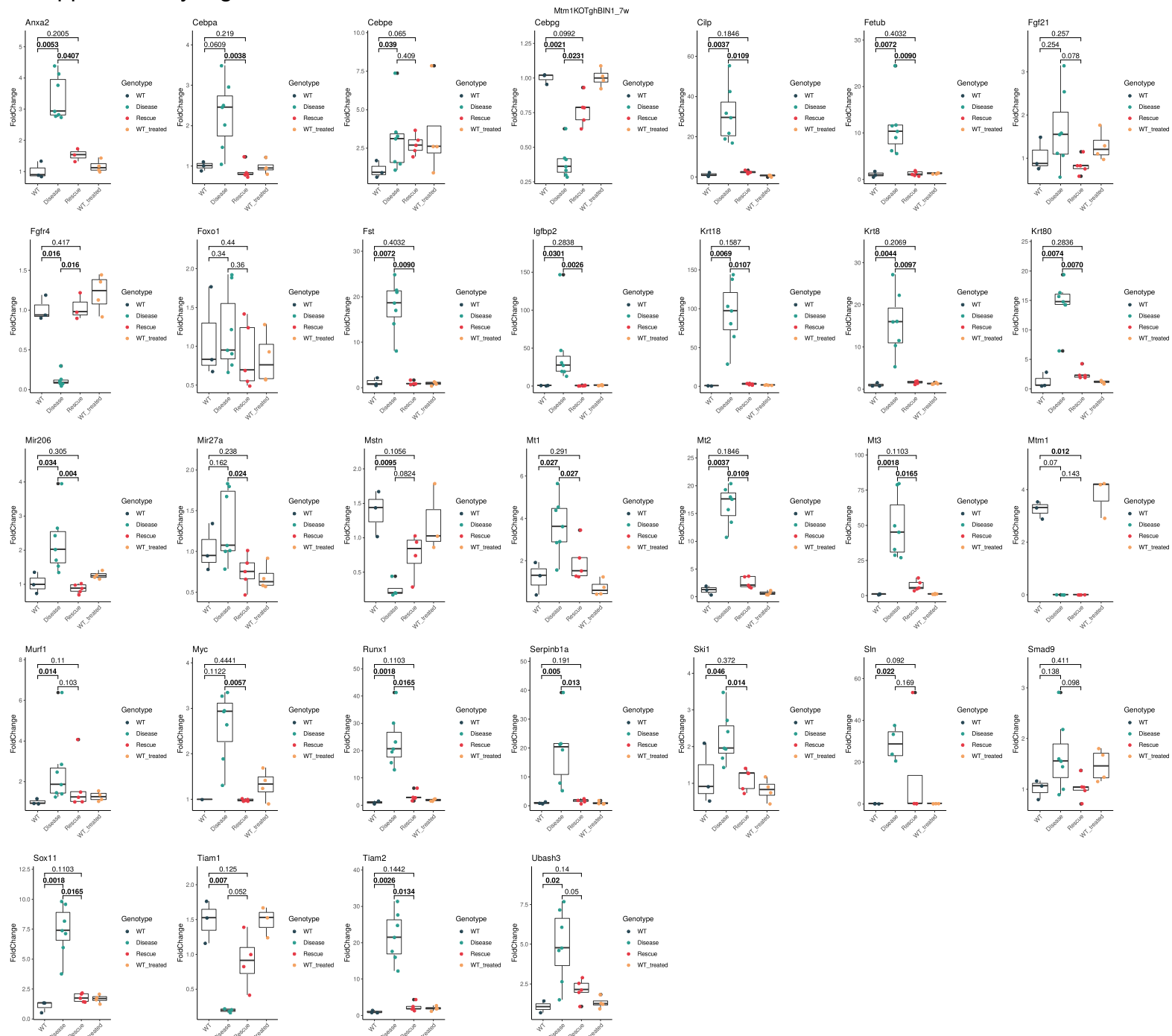
Supplementary Figure S9



Supplementary Figure S10



Supplementary Figure S11

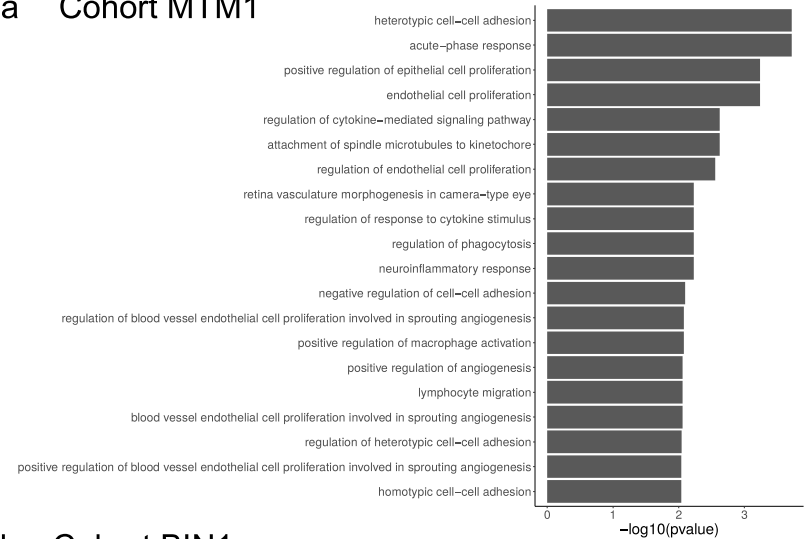


# Supplementary Figure S12

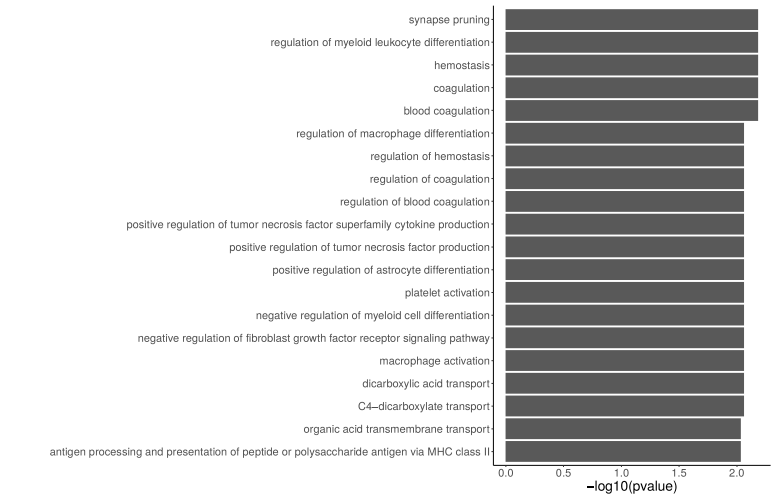


Supplementary Figure S13

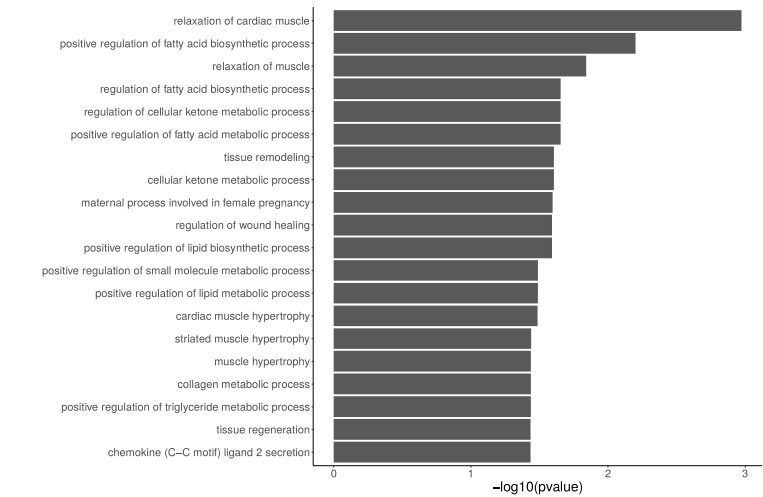
a Cohort MTM1



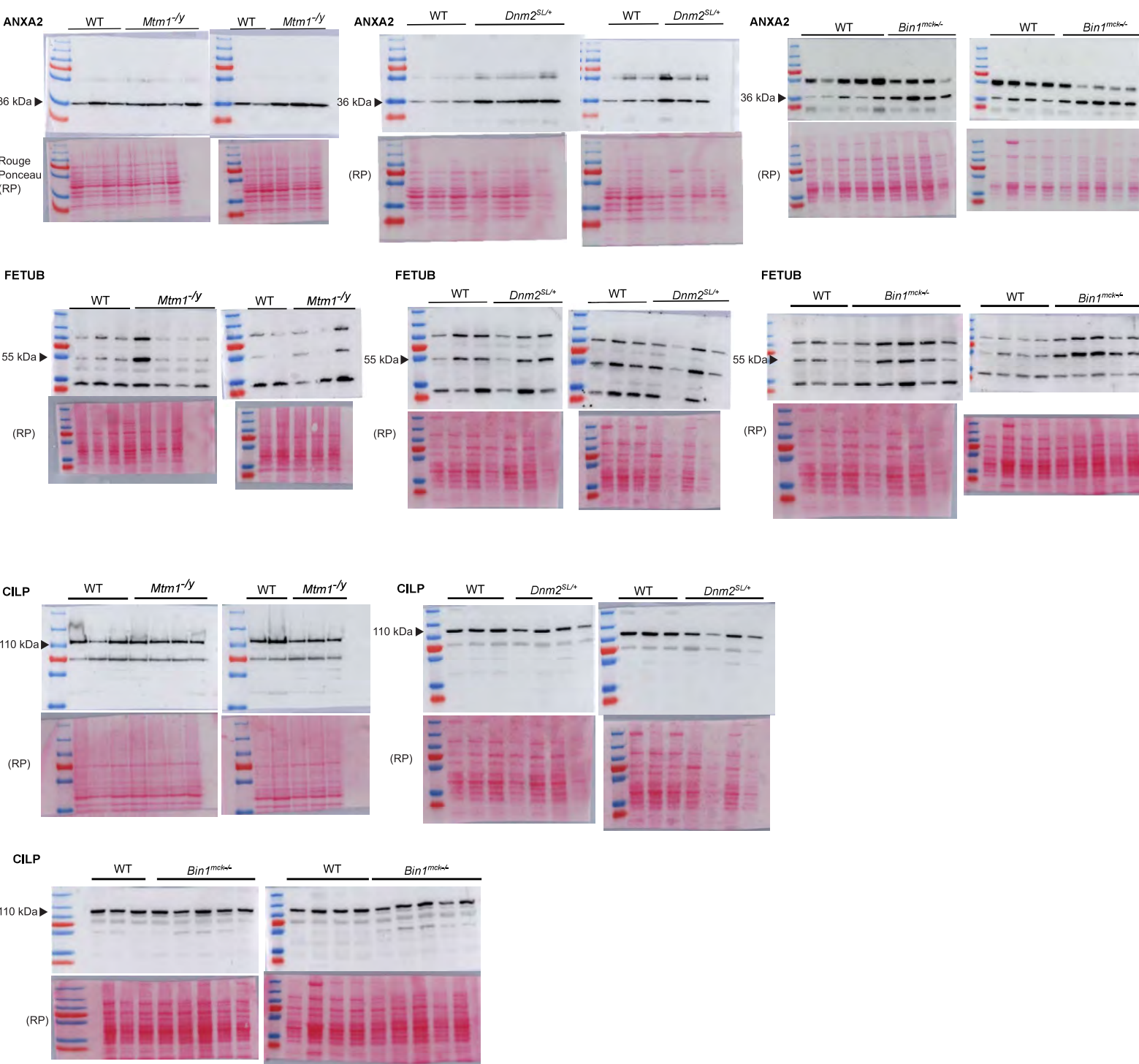
b Cohort BIN1



c Cohort DNM2



Supplementary Figure S14



Supplementary Figure S15

