

# 1 **Dysregulation of circular RNAs in myotonic dystrophy type 1**

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30

31 **Short title:**

32 Circular RNAs in DM1

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## 35 **Abstract**

36 Circular RNAs (circRNAs) constitute a recently re-discovered class of non-coding RNAs  
37 functioning as sponge for miRNAs and proteins, affecting RNA splicing and regulating  
38 transcription. CircRNAs are generated by “back-splicing”, linking covalently 3’- and 5’-ends of  
39 exons. Thus, circRNA levels might be deregulated in conditions associated to altered RNA-splicing.  
40 Indeed, increasing evidence indicates their role in human diseases. Specifically, myotonic dystrophy  
41 type 1 (DM1) is a multisystemic disorder caused by expanded CTG-repeats in the *DMPK* gene,  
42 resulting in abnormal mRNA-splicing. In this investigation, circRNAs expressed in DM1 skeletal  
43 muscles were identified by analyzing RNA-sequencing data-sets followed by qPCR validation. In  
44 muscle biopsies, out of 9 tested, 4 transcripts showed an increased circular fraction: CDYL, HIPK3,  
45 RTN4\_03 and ZNF609. The circular fraction values correlated positively with skeletal muscle  
46 strength and Receiver-Operating-Characteristics curves showed that these four circRNAs allow to  
47 distinguish DM1 patients from controls. The identified circRNAs were also detectable in  
48 peripheral-blood-mononuclear-cells (PBMCs) and plasma of DM1 patients, but they were not  
49 regulated significantly, indicating a tissue-selectivity of the identified modulations. Finally,  
50 increased circular fractions of RTN4\_03 and ZNF609 were also observed in differentiated  
51 myogenic cell lines derived from DM1 patients.

52 In conclusion, this proof-of-principle study identified circRNA dysregulation in DM1 patients.

53

## 54 **Introduction**

55 Myotonic dystrophy type 1 (DM1), also known as Steinert disease (OMIM #160900), is an  
56 autosomal dominant multi-systemic disorder, with a spectrum of clinical manifestations that include  
57 myotonia, reduced muscle strength, cardiac arrhythmia, insulin resistance, cataracts, hypogonadism,  
58 and, in the most severe forms, cognitive defects [1-4]. The genetic defect in DM1 results from the

59 dynamic expansion of CTG repeats in the 3' untranslated region of the *dystrophia myotonica*  
60 *protein kinase (DMPK)* gene [5]. Severity of the disorder generally increases with the number of  
61 CTG repeats: healthy individuals have up to 40 repeats, patients with classic DM1 have 100-1000  
62 repeats, and patients affected by congenital DM1 can have more than 2000 CTG repeats.

63 A major patho-mechanism underpinning DM1 is the generation of toxic RNAs containing  
64 expanded CUG triplets that accumulate as distinctive nuclear *foci* and dysregulate the activity of  
65 RNA processing factors, including MBNL1, CELF1, as well as Stauf1 and DDX5 [6-12].  
66 Expanded CUG repeats have been demonstrated to be toxic *per se* in several cell types and animal  
67 models [13-15], disrupting pre-mRNA alternative splicing [16]. RNA splicing alterations result in  
68 the re-emergence of developmentally immature alternative splicing and polyadenylation patterns in  
69 adult muscles, as well as in alterations of localization and turnover of specific transcripts [7,16-19].

70 Circular RNAs (circRNAs) are covalently closed loop-structure RNAs. They are generated by  
71 splicing events occurring on maturing pre-mRNAs in a different order than their genomic sequence,  
72 joining together a donor site with an upstream acceptor site [20-23].

73 CircRNAs have no accessible 5' or 3' ends and are not poly-adenylated, escaping detection by  
74 many analytical and bioinformatics tools that are widely used in RNA biology. Indeed, for a long  
75 time, circRNAs were dismissed as rare aberrant by-products of the splicing process. More recently,  
76 large RNA deep-sequencing projects and the development of bioinformatics tools enabling the  
77 analysis of extensive data-sets, allowed the identification of significant proportions of back-splice  
78 junction reads associated to circRNAs in virtually all eukaryotic organisms [24-28]. While many  
79 circRNAs likely are byproducts of RNA splicing mechanisms, some circRNAs can be even more  
80 abundant than the linear counterparts [27] and specific biological functions have been associated to  
81 a rapidly increasing number of them. Certain circRNAs contain sequences complementary to the  
82 seed of a specific microRNA (miRNA), sequestering it and therefore reducing its bioavailability for  
83 target-mRNA inhibition [29]. A prototype of this is CDR1AS/ciRS-7, a circRNA containing about

84 70 evolutionarily conserved binding sites for miRNA-7 [25,30]. Recently, CDR1AS was also found  
85 to regulate the turnover-rate of miR-7 in *Cdr1as* knock-out mice [31]. Certain circRNAs can  
86 regulate the expression of their linear counterparts, reducing the amount of pre-mRNA available for  
87 canonical splicing. Moreover, exon-intron circRNAs have also been described, that can interact  
88 with U1 snRNP and promote transcription of their parental genes [32]. Additionally, circRNAs  
89 binding and functionally interacting with RNA-binding proteins have been identified. For instance,  
90 circMBL, derived from the MBL/MBNL1 gene, in both *D. melanogaster* and humans, contains  
91 MBNL1 binding sites; MBL overexpression induces circMBL generation and this effect is  
92 dependent on the MBL binding sites [33]. Finally, a small fraction of circRNAs contains the  
93 necessary information to be translated with a cap-independent translation mechanism [26,34].

94 While it is well established that RNA splicing is aberrant in DM1, whether circRNA levels are  
95 dysregulated has not been explored yet. With this study, we provide the first evidence that the levels  
96 of specific circRNAs linked to myogenesis are deregulated in skeletal muscle biopsies and in  
97 myogenic cell cultures derived from DM1 patients. Due to their resistance to exonucleolytic  
98 degradation, circRNAs are generally more stable than linear RNAs, constituting an attractive new  
99 class of potential biomarkers [35]. Accordingly, here we also show that circRNAs are detectable in  
100 both peripheral blood mononuclear cells (PBMCs) and plasma derived from the blood of DM1  
101 patients.

102

## 103 **Methods**

### 104 **RNAseq and Bioinformatics Analysis**

105 Taking advantage of the GEO repository, we analysed RNAseq data-sets derived from *tibialis*  
106 *anterior* muscle biopsies, taken from DM1 and control patients (GSE86356) [36]. In order to ensure  
107 sufficient sequencing depth for the identification of the expectedly rare back-splicing events, we

108 used only data-sets containing more than 75 million reads (Table S1). In this way, raw reads in fastq  
109 format from 5 control and 25 DM1 ribo-depleted libraries were aligned to hg19 reference genome  
110 with BWA software (v. 0.7.12), choosing the options according to CIRI2 manual [37,38].

111 Subsequently, circRNAs were identified by detecting back-splice events in each of the aligned  
112 samples using CIRI2 (v. 2.0.6) with the suggested parameters. All identified circRNAs were then  
113 collected, normalized to each library size and quantified using custom R scripts. An abundance  
114 filter was applied by removing back-splice events present in <70% of either control or DM1  
115 samples (Table S2). The circular-to-linear ratio was calculated using the highest expressed linear  
116 junction involved in the relevant back-splice event (Table S3). Briefly, we compared all annotated  
117 linear junctions that comprised either the acceptor or the donor site of the back-splice junction and  
118 kept the one with the highest amount of spliced reads. This ensured that the ratio was determined by  
119 using two equal biological entities, i.e. reads spanning a splice junction.

## 120 **Patient characteristics and tissue collection**

121 The clinical diagnosis of DM patients was based upon the criteria set by International Consortium  
122 for Myotonic Dystrophies guidelines [39]. Genetic analysis was carried out to confirm DM1  
123 diagnosis as described previously [40]. The Muscular Impairment Rating Scale (MIRS) was used to  
124 determine the disease stage [41]. MRC scale (Medical Research Council) was used to evaluate  
125 muscle strength.

126 *Biceps brachii* muscle biopsies collected from 20 DM1 patients and 19 sex-and age matched subjects  
127 without signs of neuromuscular disorders (controls) were used for validation (Table 1).

128 PBMCs were isolated from the peripheral blood of 19 DM1 and 18 sex- and age matched controls  
129 (Table S4) by Ficoll-Paque™ PLUS (Ge Healthcare) gradient centrifugation as described before  
130 [42]. The plasma of 29 DM1 and 28 age-and sex- matched controls (Table S4) was collected in  
131 EDTA-tubes and cells as well as platelets were removed as described previously [43,44].

<b>Clinical Characteristics</b>	<b>DM1 (n=20)</b>	<b>CTRL (n=19)</b>
<b>Age at sampling (average <math>\pm</math> se)</b>	40.9 $\pm$ 3.3	38.6 $\pm$ 1.1
<b>Sex (male/female)</b>	10/10	15/4
<b>MRC megascore (average <math>\pm</math> se)</b>	121.6 $\pm$ 1.6	130 $\pm$ 0.0
<b>Myotonia (% of patients)</b>	70	0
<b>Glucose (normal values: 70–110 mg/dl)</b>	89.9 $\pm$ 5.5	89.0 $\pm$ 3.8
<b>Cholesterol (normal values: &lt; 200 mg/dl)</b>	213.8 $\pm$ 13.2	N. A.
<b>CK</b>	Male: 247.7 $\pm$ 42.7	N. A.
<b>(normal values: male &lt; 190 mg/dl. female &lt; 125 mg/dl)</b>	Female: 213.1 $\pm$ 63.2	N. A.
<b>Arrhythmia (% of patients)</b>	15.4	0
<b>Cataract (% of patients)</b>	7.7	0
<b>ECG-QRS duration (normal values: 60–110 ms)</b>	99.4 $\pm$ 3.7	N. A.
<b>Number of CTG repeats (range)</b>	490.6 $\pm$ 64.1 (90-1100)	N. A.
<b>Stage of disease (range 1–5)</b> <b>(% of patients at each stage)</b>	Stage 1: 0	N.R.
	Stage 2: 35.3	
	Stage 3: 17.6	
	Stage 4: 41.2	
	Stage 5: 5.9	

132 **Table 1. Clinical data on DM1 and control patients used for validation in biopsies.** N.R.: not  
 133 relevant. N.A.: not available.

134

## 135 **Ethical approval and informed consent**

136 The experimental protocol was reviewed and approved by the Institutional Ethics Committee of  
 137 the San Raffaele Hospital (protocol miRNADM of 23.06.2015) and was conducted according to the

138 principles expressed in the Declaration of Helsinki, the institutional regulation and the Italian laws  
139 and guidelines. A written informed consent was obtained from each patient prior to muscle biopsies  
140 or blood collection.

## 141 **Histopathological analysis**

142 Muscle tissue was fresh-frozen in isopentane cooled in liquid nitrogen. Histopathological analyses  
143 were performed on serial sections (8  $\mu\text{m}$ ) processed for routine histological or histochemical  
144 stainings. Myofibrillar ATPase staining was performed as previously described, after sample pre-  
145 incubation at pH 4.3, 4.6, and 10.4 [45].

## 146 **DM1 myogenic cell lines**

147 Immortalized human myotonic dystrophy muscle cell lines expressing murine *Myod1* cDNA  
148 under the control of a Tet-on inducible construct were previously described [46]. Cells were  
149 cultivated in DMEM growth medium (15% FBS) until confluency was reached. Myogenic  
150 differentiation was induced by switching cell cultures to DMEM supplemented with 5  $\mu\text{g}/\text{ml}$  insulin  
151 and 4  $\mu\text{g}/\text{ml}$  doxycycline (Sigma-Aldrich).

152 For silencing experiments, cells were transfected after 3 days of differentiation with 50nM  
153 MBNL1/CELF1 TARGETplus SMARTpool siRNAs (Dharmacon) or with 50nM ON-  
154 TARGETplus Non-targeting Pool as negative control. Cells were transfected using HiPerFect  
155 reagent (Qiagen), according to manufacturer's instructions. Three days after transfection, total RNA  
156 was isolated and analyzed by qPCR.

## 157 **Isolation of total RNA**

158 Total RNA was extracted from the muscle tissues using TRIzol reagent (Thermo Fisher Scientific  
159 Inc.) as described previously [47,48]. For isolation of total RNA from PBMCs and cells, TRIzol  
160 reagent was used according to the manufacturer's instructions. The purity and integrity of the  
161 obtained RNAs were measured by Nanodrop (Thermo Fisher Scientific Inc.).



162 Total RNA from plasma samples was extracted as previously described using NucleoSpin miRNA  
163 Plasma columns (Macherey-Nagel) [43,44].

## 164 **Real-time reverse transcriptase qPCR**

165 For validation experiments, total RNA was first retro-transcribed using the SuperScript Reverse  
166 Transcriptase kit (version III or IV) and then investigated by SYBR green qPCR, according to the  
167 manufacturer's protocol (Thermo Fisher Scientific Inc.). Primer couples were designed by Primer-  
168 BLAST tool (Table S5).

169 The relative expression was calculated using the comparative Ct method  $2^{-\Delta\Delta CT}$  [49], normalizing  
170 to the averaged Cts of RPL13, RPL23 and UBC for tissue RNAs and to the averaged Cts of miR-  
171 106a and miR-17-5p for plasma RNAs [43,44].

172 The circular-to-linear ratio was estimated by subtracting the raw Ct of the linear transcript from  
173 the raw Ct of the corresponding circular transcript.

174 For score-calculations, the log<sub>2</sub> fold changes of all significantly modulated circRNAs or circular-  
175 to-linear ratios were averaged.

## 176 **Statistical Analysis.**

177 GraphPad Prism 7.01 (GraphPad Software Inc.) was used for statistical analysis and for graph  
178 generation. Following differential expression analysis, all data-sets were checked for their  
179 distribution by D'Agostino & Pearson normality test. The only exception were the cell line data,  
180 since due to the small sample size normality test was not possible. The differential expression of  
181 circRNAs in biopsies was investigated by multiple t-test, using the recommended settings of  
182 GraphPad for false discovery rate (Benjamini, Krieger and Yekutieli) and Q= 1% as significance  
183 cut-off. For differential expression analysis of circRNAs in PBMCs, plasma and cell lines, two-  
184 tailed Student's t test or Mann Whitney was used, depending on the data-distribution. A p <0.05 was  
185 deemed statistically significant. Values are expressed as  $\pm$ standard error.

186

## 187 **Results**

### 188 **Identification of circRNA expression in DM1 skeletal muscle by** 189 **RNA-sequencing.**

190 Published RNA sequencing (RNAseq) data of ribo-depleted libraries derived from 5 controls and  
191 25 DM1 *tibialis anterior* biopsies [36] were investigated for circRNA expression. Among the  
192 available data-sets, we analyzed only libraries containing at least 76 million reads, thus providing  
193 sequence information at a high depth (Table S1). By applying CIRI2 [37,38], an algorithm designed  
194 for the discovery of circRNAs, a total of 21.822 unique back-splice sites were identified across all  
195 libraries. Since most of these events were present in few samples only, a stringent abundance filter  
196 was used, resulting in  $\approx 1.800$  back-splice junctions (Table S2). Certain circRNAs display  
197 expression levels comparable to or even higher than their linear counterparts, suggesting a potential  
198 biological relevance [27]. To identify these particularly interesting circRNAs, the circular-to-linear  
199 ratios were estimated. Therefore, all annotated linear junctions involved with either the donor or the  
200 acceptor site of the back-splice event were quantified. The linear junction with the highest  
201 expression was then compared to the back-splice junction, revealing 578 circRNAs with a circular-  
202 to-linear ratio  $>0.5$  in DM1 (Table S3).

203 To narrow the list of candidates for validation, the  $\approx 1.800$  identified circRNAs were intersected  
204 with a list of 29 circRNAs that were previously validated in human and mouse myoblasts [26],  
205 resulting in 18 common circRNAs (Table S3). Interestingly, most of them displayed a circular-to-  
206 linear ratio  $>0.5$ .

### 207 **Validation by qPCR of differentially expressed circRNAs in DM1** 208 **skeletal muscles.**

209 Muscle tissue biopsies were harvested from *biceps brachii* of 20 DM1 and 19 sex- and age-  
210 matched control individuals, with no sign of neuromuscular disorders. The DM1 group showed the  
211 main characteristics of the disease, such as myotonia, cataract and muscle weakness (Table 1), as  
212 well the typical histological alterations, such as central *nuclei*, high variation in fiber size, atrophic  
213 fibers and nuclear clumps (Fig. S1) [1,39]. Most DM1 patients were at stage 2-4 and the  
214 pathological expansions of the CTG triplets ranged from 90 to 1100.

215 Total RNAs were isolated and the expression of a set of circRNAs and their linear counterparts  
216 was measured by qPCR. Out of the 18 circRNA candidates, 8 primer couples passed all technical  
217 checks of specificity and efficiency. Of note, two circRNAs originated from the same gene and  
218 were indicated as circRTN4 and circRTN4\_03. The primers designed for circRNAs produced an  
219 amplicon spanning the back-splice junction, while the linear primers resulted in amplicons crossing  
220 the linear junction to a neighboring exon. Due to the key role of MBNL in DM1, the previously  
221 identified circRNA hosted in the second exon of MBNL [33] and its linear form were also  
222 measured. Thus, a set of 9 circRNAs together with their linear counterparts were used for validation  
223 (Table S4). All tested transcripts were confirmed to be readily expressed also in *biceps brachii*  
224 biopsies, with the only exception of circMBNL1 (circMBNL1), showing an expression close to the  
225 detection threshold. Five circRNAs, circASPH, circCDYL, circHIPK3, circRTN4\_03 and  
226 circZNF609, displayed a statistically significant increase following multiple comparison testing  
227 ( $q < 0.01$ ) (Fig. S2).

228 To assess whether the observed induction of the circular transcripts was simply the consequence  
229 of a general increase of transcription in the relevant genomic region in DM1 patients, modulation of  
230 the ratios between the circular and the linear isoforms was calculated. We identified 4 circRNAs  
231 (circCDYL, circHIPK3, circRTN4\_03 and circZNF609) with a significantly increased, circular-to-  
232 linear ratio in DM1 muscles (Fig. 1a), implying a de-regulation of the circular transcript  
233 independent from its linear counterpart. Accordingly, a similar trend was also observed in the

234 RNAseq data, where the circular-to-linear ratios were higher in the DM1 affected muscles  
235 compared to the controls (Table S3).

236

237 **Figure 1. Differentially modulated circular-to-linear ratios in DM1 skeletal muscles. (a)**

238 Scatterplots in log<sub>2</sub> scale of significantly different circular-to-linear ratios identified by qPCR in  
239 DM1 muscle tissue compared to control (CTRL). After normality test, statistical significance was  
240 calculated either by t-test or Mann-Whitney test (threshold  $p < 0.05$ ), followed by correction multiple  
241 comparison, with significance threshold set at  $q < 0.01$ . **(b)** Scatterplot of circular-to-linear ratio,  
242 estimated by averaging the log<sub>2</sub> fold changes of significantly different circular-to-linear ratios in  
243 DM1 muscle tissue compared to control. For both panels, lines indicate mean and standard error  
244 values for each group. DM1= 20 (red dots); CTRL= 19 (black dots); \*\* $q < 0.001$ ; \*\*\* $p < 0.001$ .

245

246 **DM1-circRNAs distinguish DM1 patients from controls.**

247 To understand if the identified DM1-deregulated circRNAs (DM1-circRNAs) display a  
248 discriminating power to identify DM1 patients, Receiver Operating Characteristic (ROC) curve  
249 analysis was performed. Both, significantly increased circRNAs alone (Fig. S3) and circular-to-  
250 linear ratios (Fig. 2) were analyzed. Interestingly, with one exception (circCDYL), the  
251 discrimination power between diseased and healthy individuals increased using the circular-to-  
252 linear ratios. In detail, among the five tested ratios, ZNF609 showed the largest area under the curve  
253 (AUC= 0.92), while the others ranged between 0.84 and 0.86 (Fig. 2a). Intriguingly, averaging all  
254 five DM1-circRNA fractions into a “circular-to-linear score” (Fig. 2b) improved the performance  
255 (AUC= 0.89) with respect to the singular fractions, with the exception of circZNF609, continuing to  
256 show the largest AUC (Fig. 2a).

257 In conclusion, each of the five circular-to-linear ratios, as well as the combined “circular-to-linear  
258 score” of the DM1-circRNAs are useful to discriminate healthy from diseased patients.

259

260 **Figure 2. Discrimination of DM1 patients from controls using circular-to-linear ratios of**  
261 **DM1-circRNAs. (a)** ROC curves show the sensitivity and specificity of each circRNA fraction  
262 (circ/lin) and of the combined “circular-to-linear score” to distinguish DM1 from healthy muscle  
263 tissue. **(b)** The “circular-to-linear score” was calculated by averaging the significantly modulated  
264 circular-to-linear ratios (circ/lin score, black rectangle). DM1= 20, CTRL= 19.

265

266 **Correlation between DM1-circRNAs and clinical characteristics.**

267 To evaluate a potential relationship between the deregulation of circRNAs and clinical conditions,  
268 correlation analyses were performed. One of the most clinically relevant parameters for DM1  
269 patients is muscle strength, measured by the Medical Research Council (MRC) grading system. We  
270 found that the changes of the circular fractions of circCDYL, circHIPK3, circRTN4\_03 and  
271 circZNF609 displayed a significant negative correlation to MRC (Fig. 3a). Accordingly, a negative  
272 correlation was observed also between the MRC grading and the circular-to-linear score (Fig. 3b).  
273 The strongest and most significant correlation was found for the circular fraction of ZNF609, with  
274 Pearson  $r= 0.57$  and  $p=0.0002$ .

275 Collectively, these data suggest a potential of circRNAs as DM1 biomarkers, in spite of the low  
276 number of subjects analyzed.

277

278 **Figure 3. Correlation of muscle strength with circular-to-linear ratios of DM1-circRNAs. (a)**  
279 Pearson’s correlation between significantly modulated circular-to-linear ratios identified in skeletal  
280 muscle biopsies and muscle strength measured by MRC megascore. **(b)** Pearson’s correlation of the

281 “circular-to-linear score” (obtained averaging all DM1-circRNA fractions) and muscle strength  
282 measured by MRC megascore. DM1= 20 (red dots), CTRL= 19 (black dots).

283

## 284 **DM1-circRNA levels in PBMCs and plasma of DM1 patients.**

285 Since peripheral blood can be obtained with a minimally invasive procedure, it represents a  
286 potentially interesting tissue for biomarker identification. Thus, we measured DM1-circRNA  
287 expression in PBMCs and plasma of DM1 patients and sex- and age-matched controls. Diseased  
288 and healthy subjects were chosen with the same criteria adopted for the harvesting of skeletal  
289 muscle biopsies (Table S4).

290 All DM1-circRNAs were readily detectable in PBMCs, but none of them showed a significant  
291 modulation (Fig. S4).

292 Among the DM1-circRNAs tested in plasma samples, circCDYL and circRTN4 were readily  
293 detectable. A small, but not significant induction could be observed in DM1 patients for circCDYL  
294 and circRTN4, consistent with the data obtained in biopsies (Fig. S5).

295 We conclude that the DM1-circRNA dysregulations observed in skeletal muscles are tissue  
296 restricted.

## 297 **DM1-circRNA expression in DM1 myogenic cell lines**

298 We assessed whether circRNA alterations identified in DM1 muscle biopsies were also observed  
299 in cultured myoblasts. To this aim, we took advantage of DM1 and control muscle cell lines  
300 obtained by conversion of immortalized skin fibroblasts into multinucleated myotubes by forced  
301 expression of *MyoD1* [46]. In DM1 and control differentiated myogenic cells, all circRNAs tested  
302 were readily detectable. Interestingly, two of the circular transcripts, circRTN4 and circRTN4\_03  
303 were significantly increased in DM1 compared to control (Fig. S6). The analysis of their circular-to-  
304 linear ratio confirmed the induction of circRTN4\_03. Additionally, an increased circular-to-linear

305 ratio was also observed for ZNF609. These results are in line with the findings obtained in DM1  
306 biopsies (Figure 4).

307 We took advantage of this cell culture system to investigate whether silencing of DM1-related  
308 splicing factor affected the levels of the DM1-circRNAs modulated *in vitro*.

309 Since MBNL1 is impaired in DM1 patients [7,9,10], we assayed whether MBNL1 silencing in  
310 control myogenic cells induced, at least in part, the circRNA deregulations observed in DM1  
311 myogenic cells. Control differentiated myogenic cells were transfected with MBNL1 siRNAs or  
312 relevant control oligonucleotides and RNA was extracted 3 days later. MBNL1 mRNA was  
313 significantly down modulated (Fig. S7a) and the expected alterations in the alternative-splicing  
314 patterns of SERCA1 and IR (Insulin Receptor) were observed (Fig. S7b and c) [50,51]. However, no  
315 increase was observed in the abundance of circZNF609, circRTN4 and circRTN4\_03 levels (Fig.  
316 S7d).

317 We also tested whether the silencing of CELF1, which is activated in patients and in DM1 disease  
318 models [52], rescued DM1-circRNA expression in DM1 differentiated myogenic cells. In spite of  
319 effective CELF1 knock-down (Fig. S8a), no significant change of circZNF609, circRTN and  
320 circRTN\_03 levels was observed (Fig. S8b).

321 We conclude that the DM1 myogenic cell lines studied reflect, at least in part, the outcome of the  
322 DM1 biopsies and therefore represent a valuable tool for functional studies *in vitro*.

323

324 **Figure 4. Differentially modulated circular-to-linear ratios in myogenic cell lines.** Boxplots of  
325 significantly different circular-to-linear ratios identified by qPCR in differentiated DM1 myogenic  
326 cells compared to controls (\* $p < 0.05$ ; \*\* $p < 0.01$ ; DM1= 4; CTRL= 4).

327

328

## 329 **Discussion**

330 A molecular hallmark of DM1 is the dysregulation of alternative splicing, affecting many genes  
331 involved in muscle homeostasis and function [16,17,53]. CircRNAs are indeed alternative splicing  
332 products [20,21] and, in this proof-of-principle study, we provide evidence of de-regulation of  
333 circRNA expression in DM1 patients. We analyzed 30 publicly available gene-expression data-sets  
334 of DM1 and control *tibialis anterior* muscles [36], using bioinformatics tools designed for the  
335 identification of circRNA-specific back-splice events. Applying stringent selection and abundance  
336 filters, we identified  $\approx 1.800$  unique circular splicing events, a number comparable to circRNAs found  
337 to be expressed in human myoblasts and myotubes [26].

338 During the testing phase of the bioinformatics pipeline with other data-sets, we performed several  
339 attempts of differential expression analysis (data not shown). Unfortunately, performances obtained  
340 were largely unsatisfactory, likely due to very low read numbers for most circRNA species and to  
341 normalization difficulties. Therefore, in this study we chose another approach for the identification  
342 of circRNAs potentially relevant in DM1. We filtered for circRNAs displaying expression across  
343 many samples and then selected circRNAs previously shown to be involved in myogenesis [26]. Of  
344 note, most of these circRNAs also displayed a high circular-to-linear ratio. This suggests that these  
345 circRNAs are not a mere by-product of the transcript maturation process, and might also indicate an  
346 independent regulation as well as an additional biological function.

347 For opportunity reasons, the validation step was performed in *biceps brachii* biopsies, since only  
348 for this muscle type a sufficient number of samples was available to us. It should be acknowledged  
349 that *biceps brachii* is generally less severely affected than other distal muscles in DM1 patients [1-  
350 3,39]. Thus, some of the circRNA level differences that failed to reach statistical significance in our  
351 validation analysis, might be indeed relevant in distal muscles. On the other side, it is plausible to  
352 hypothesize that circRNA alterations identified in proximal muscles could be more pronounced in  
353 distal muscles.



354 In spite of the preliminary and not-comprehensive nature of this study, we found that the levels of  
355 5 out of 9 circRNAs tested were significantly increased, suggesting a potentially pervasive  
356 dysregulation of circRNAs in DM1. Accordingly, 4 of these circRNAs also displayed an increased  
357 circular-to-linear ratio. The most likely interpretation of these alterations is that they are caused by  
358 the dysfunction of the alternative splicing machinery characterizing DM1 [16,17,53]. Silencing of  
359 either MBNL1 or CELF1 in control and DM1 cultured myotubes did not affect the abundance of  
360 circZNF609, circRTN4 and circRTN4\_03. While negative data should always be evaluated in a  
361 very cautious manner, one possible interpretation is that other splicing factors, such as different  
362 MBNL-family members, Staufen1 and DDX5 [6-12], regulate the generation of the DM1-  
363 circRNAs. Moreover, these splicing factors might be redundant in circRNA regulation, making the  
364 silencing of a single splicing factor ineffective. Finally, higher stability of circRNAs compared to  
365 their linear counterparts [54] should also be considered as possible mechanism underpinning the  
366 increase of the circular-to-linear ratio of DM1-circRNAs.

367 CircRNA dysregulation may lead to pathological consequences to be investigated. In this respect,  
368 however, little is known of the identified DM1-circRNAs. CircHIPK3 positively regulates human  
369 cell growth by sponging multiple miRNAs [55,56]. Among these miRNAs, there are miR-29b and  
370 miR-193a. Intriguingly, both miRNAs were previously found to be down-modulated in DM1 [57]  
371 and DM2, respectively [47]. CircHIPK3 levels are also increased in retinal endothelial cells  
372 exposed to diabetes-related stressors, in retinas of diabetic mice and in the plasma of diabetic  
373 patients. Moreover, in retinal endothelial-cells, circHIPK3 affects cell viability, proliferation,  
374 migration, and function [58]. While the implication of circHIPK3 in DM1 should be investigated, it  
375 is worth noting that insulin resistance is very often present in DM1 patients [1-3,59].

376 CircZNF609 is downregulated during myogenesis and can specifically control myoblast  
377 proliferation [26]. Moreover, its mouse homologue circZfp609 suppresses myogenic differentiation  
378 [60]. Of note, circZNF609 is elevated in Duchenne muscular dystrophy myoblasts, indicating that

379 the DM1-circRNAs identified in this study might be deregulated in other muscular diseases. Like  
380 circHIPK3, also circZNF609 is induced by high glucose *in vivo* and *in vitro* and regulates the  
381 function of retinal endothelial cells [61].

382 Finally, the host gene of circRTN4 displays muscle-specific splicing [62]. It is a direct target of  
383 RBM20, an alternative-splicing regulator of cardiac genes, associated with coronary heart disease.  
384 Mutation of RBM20 results in altered splicing of its target genes, causing the retention of specific  
385 exons of RTN4 mRNA. Since RBM20 is also expressed and active in skeletal muscles [63], further  
386 investigations are needed to assess the potential involvement of RBM20 also in circRTN4  
387 formation.

388 Ashwal-Fluss and collaborators [33], by studying circRNAs identified in neuronal tissues,  
389 reported that the second exon of the splicing factor *MBLNI* (*MBL* in drosophila) is circularized in  
390 flies and humans and that circMBL production competes with canonical pre-mRNA splicing.  
391 Moreover, Muscleblind protein interacts with flanking introns of its own gene to promote exon  
392 circularization. This observation prompted us to measure circMBNL1 in skeletal muscle biopsies  
393 and PBMCs of DM1 patients, where MBNL1 protein bioavailability is reduced by its sequestration  
394 in nuclear CUG-foci [9,10]. However, we did not observe any modulation of circMBNL1 in these  
395 tissues. The most likely explanation is that circMBNL1 regulation is highly context specific.  
396 Indeed, while in fly heads, this circRNA is more abundant than the linear counterpart, the opposite  
397 seems to be true in human skeletal muscle, where RNAseq data indicated a circular-to-linear ratio  
398 of 0.05 (Table S3). In keeping with this hypothesis, very low circMBL levels were also observed in  
399 drosophila S2 cells [33]. Furthermore, changes in the splicing pattern of MBNL1 mRNA  
400 (comprising or not exon1) were observed in cardiac and skeletal muscles, but not in the brain of  
401 DM1 patients compared to controls [64,65], confirming a high tissue-specificity in the regulation of  
402 MBNL1 transcript.

403 Identification of diagnostic and prognostic biomarkers is an unmet clinical need for DM1 patients.  
404 Recent studies suggest that alternative splicing isoforms in skeletal muscle tissue have a high  
405 potential as biomarkers of DM severity and for the monitoring of therapeutic responses [53]. Due to  
406 their loop-structure, circRNAs are highly resistant to exonucleases [54], holding a great potential as  
407 disease biomarkers. Promisingly, the potential of circRNAs as molecular markers has been  
408 highlighted recently in various types of cancer [66], measuring circRNAs not only in biopsies of  
409 solid tissues, but also in extracellular compartments, such as serum or exosomes [66,67].

410 It is still too early to conclude whether circRNAs will find their way to the clinic. This will largely  
411 depend on both technical issues, such as detectability and stability of circRNAs in biological  
412 samples, as well as on whether circRNAs are functional elements of the molecular mechanisms  
413 driving the disease or mere byproducts [54]. While further studies are obviously needed, we found  
414 that the circular-to-linear ratios as well as the combined “circular-to-linear score” of the DM1-  
415 circRNAs in skeletal muscle biopsies accurately discriminated healthy from DM1 patients.  
416 Moreover, a correlation between the MRC grading and the circular-to-linear score could be  
417 identified. Additional investigations are needed to evaluate the potential of circRNAs as DM1  
418 biomarker, involving a higher number of subjects. Comparison with other myopathies will also  
419 allow to investigate their possible disease-specificity.

420 CircRNAs are detectable also in the peripheral blood that can be harvested with minimally-  
421 invasive techniques. Indeed, DM1-circRNAs were measured both in PBMCs and, at least in part, in  
422 plasma samples. On the one side, the high context-dependent regulation of circRNAs implicates  
423 that the pattern of expression observed in the peripheral blood is unlikely to mirror that of other  
424 tissues, such as the skeletal muscle. On the other side, the apparent pervasiveness of circRNA  
425 dysregulation suggest that comprehensive screenings of circRNAs might indeed succeed in the  
426 identification of common circRNAs between blood and affected organs. An alternative approach

427 may be represented by the analysis of urine extracellular RNAs, whose splice variants have been  
428 shown to discriminate DM1 patients efficiently [68].

## 429 **Acknowledgments**

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433 clinical data collection.

434

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601

## 602 **Supporting information**

603 **Figure S1. Histopathological analysis of *biceps brachii* biopsies obtained from a representative**  
604 **DM1 patient (a, b) and from a representative control (c).** In DM1 patient, Hematoxylin & Eosin  
605 **(a)** and ATPase pH 10.4 **(b)** stainings displayed the characteristic histopathological features of DM1,  
606 such as central nuclei (asterisks), atrophic fibers (arrow) and an evident fiber size variability both of  
607 type 1 (negative fibers) and type 2 (brown fibers) fibers.

608 **Figure S2. Significantly modulated circRNAs in DM1 skeletal muscles.** Scatterplots of circRNA  
609 (circ.) transcripts identified by qPCR as differentially expressed in DM1 *biceps brachii* biopsies are  
610 shown together with their linear counterparts (lin.). Lines indicate mean and standard error values for  
611 each group. After normality test, statistical significance was calculated either by t-test or Mann-  
612 Whitney test (threshold  $p < 0.05$ ), followed by correction multiple comparison, with significance  
613 threshold set at  $q < 0.01$  (\* $q < 0.01$ ; \*\* $q < 0.001$ ). DM1= 20 (red dots), Controls= 19 (CTRL, black dots).

614 **Figure S3. Discrimination of DM1 from healthy patients by circRNA modulation.** ROC curves  
615 show the sensitivity and specificity of each DM1-circRNA to distinguish DM1 from healthy muscle  
616 tissue. DM1= 20, CTRL= 19.

617 **Figure S4. DM1-circRNA levels in PBMCs derived from DM1 patients and controls.** RNA was  
618 extracted from PBMCs of DM1 and control individuals. Scatterplots show the levels of DM1-  
619 circRNAs detected by qPCR. DM1= 19 (red dots), Controls= 18 (CTRL, black dots). Lines indicate  
620 mean and standard error values for each group.

621 **Figure S5. DM1-circRNA levels in plasma derived from DM1 patients and controls.** RNA was  
622 extracted from platelet-free plasma of DM1 and control individuals. Scatterplots show the levels of  
623 circRNAs detectable by qPCR. DM1= 29 (red dots), Controls= 28 (CTRL, black dots). Lines indicate  
624 mean and standard error values for each group.

625 **Figure S6. Significantly modulated circRNAs in DM1 myogenic cell lines.** Boxplots of  
626 differentially expressed circRNAs (circ.) and their linear counterparts (lin.) identified by qPCR in  
627 differentiated DM1 myogenic cells compared to controls (\* $p < 0.05$ ; \*\* $p < 0.01$ ; DM1= 4; CTRL= 4).

628 **Figure S7. MBNL1 silencing does not affect DM1-circRNA levels.** Differentiated control  
629 myogenic cells were transfected with siRNAs targeting MBNL1 or with control siRNAs (n=5). (a)  
630 Efficiency of MBNL1 knock-down was assessed by qPCR (\*\* $p < 0.0001$ ). (b and c) MBNL1  
631 silencing induced the expected increases of the SERCA1 isoform excluding exon 22 (isoform b),  
632 and of the IR isoform excluding exon 11 (isoform a), as assessed by PCR followed by agarose gel  
633 electrophoresis. Representative gels are shown. (d) circZNF609, circRTN4 and circRTN4\_03 levels  
634 were measured by qPCR. None of the circRNAs displayed a statistically significant increase.

635 **Figure S8. CELF1 silencing does not affect DM1-circRNA levels.** Differentiated DM1 myogenic  
636 cells were transfected with siRNAs targeting CELF1 or with control siRNAs (n=3). (a) Efficiency of  
637 CELF1 knock-down was assessed by qPCR (\*\* $p < 0.0001$ ). (b) circZNF609, circRTN4 and  
638 circRTN4\_03 levels were measured by qPCR. None of the circRNAs displayed a statistically  
639 significant decrease.

640

641 **Table S1. Library size of publicly available data-sets used for circRNA identification in DM1**  
642 **skeletal muscle by RNAseq.** A set of 30 transcriptomes (25 DM1 and 5 healthy controls) from human  
643 tibialis biopsies (GSE86356) was investigated for back-splice events. The sequencing depth is  
644 reported as million sequenced reads after mapping to the human genome version hg19. Each data-set  
645 is identified by its SRA Run number; controls are highlighted in grey.

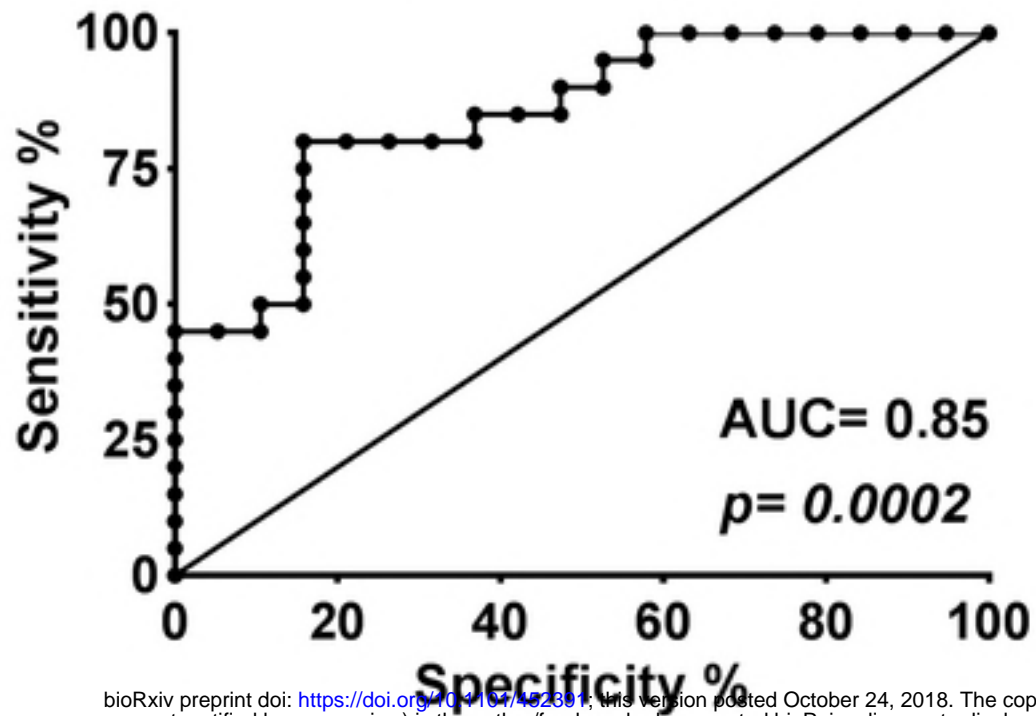
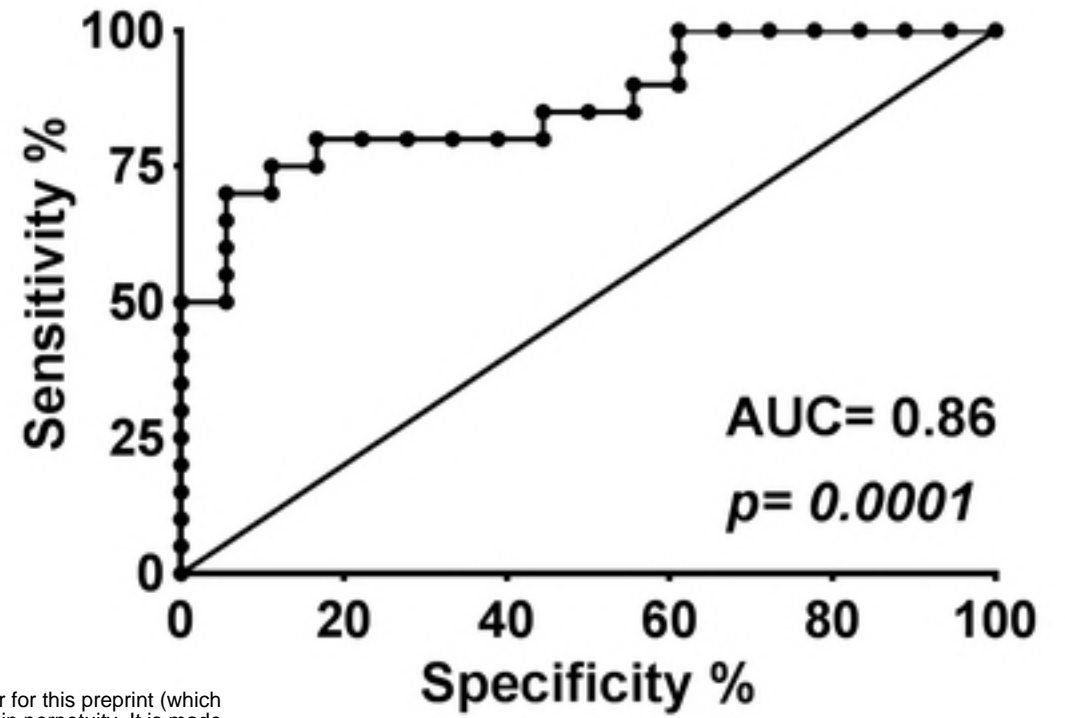
646 **Table S2. circRNA identification and expression in DM1 skeletal muscle by RNAseq.**  
647 Normalized counts of 1797 different circRNA species identified by CIRI2-algorithm, following a  
648 filtering step for abundance. Each back-splice junction is identified with the coordinates of the

649 involved donor and acceptor sites in the format “chromosome number: donor position | acceptor  
650 position”. Each data-set is identified by its SRA Run number, controls are highlighted in grey.

651 **Table S3. Ratios of circular versus linear expression levels measured in DM1 skeletal muscle**  
652 **by RNAseq.** For estimation of circular-to-linear ratios, the linear junction with the highest coverage  
653 involved with either the donor or the acceptor site of the back-splice event was determined. The  
654 averaged, normalized counts across all libraries in each condition were calculated for linear and back-  
655 splice junction. The circular-to-linear ratios were determined for controls and DM1 and are here  
656 highlighted in grey. Additionally, the circRNAs identified in human tibialis biopsies were intersected  
657 with myogenic circRNAs identified by Legnini et al. [26] The final validation set chosen for qPCR  
658 is displayed in the column “Validation-set”.

659 **Table S4. Clinical data of DM1 patients and controls used for circRNA detection in plasma**  
660 **and PBMCs.** NR: not relevant, NA: not available.

661 **Table S5. Primer-sequences.** List of primer-couples used for relative quantification of circRNAs  
662 and their linear counterparts by qPCR. With the exception of CDYL and HIPK3, circular transcripts  
663 and their linear counterparts shared one primer, either forward or reverse.

**a***circ/lin* CDYL*circ/lin* HIPK3

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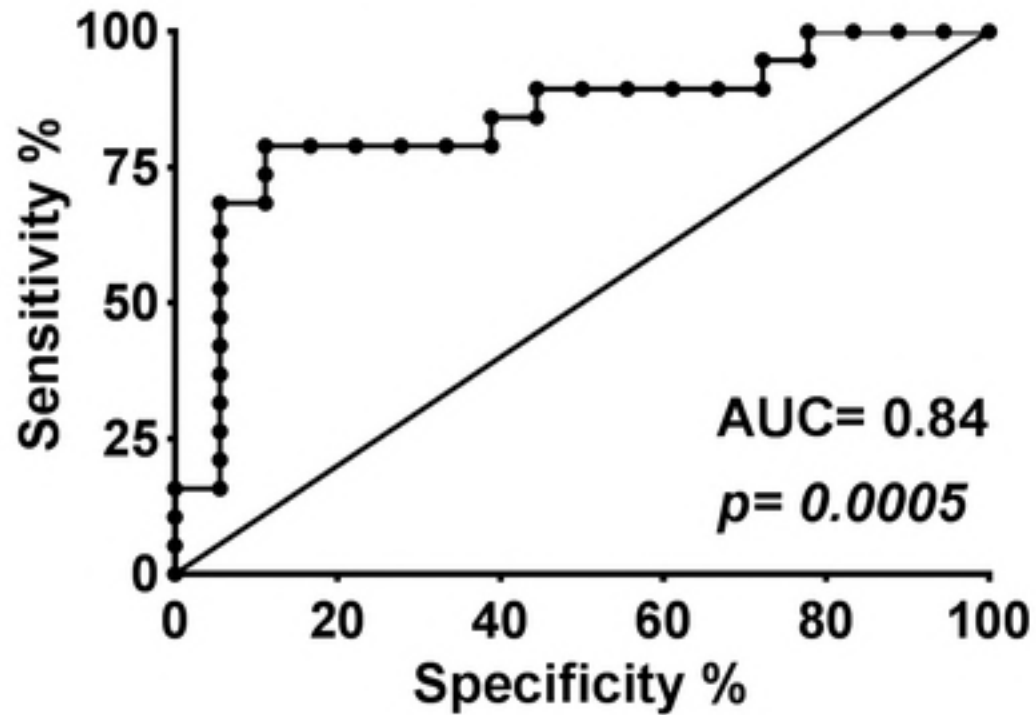
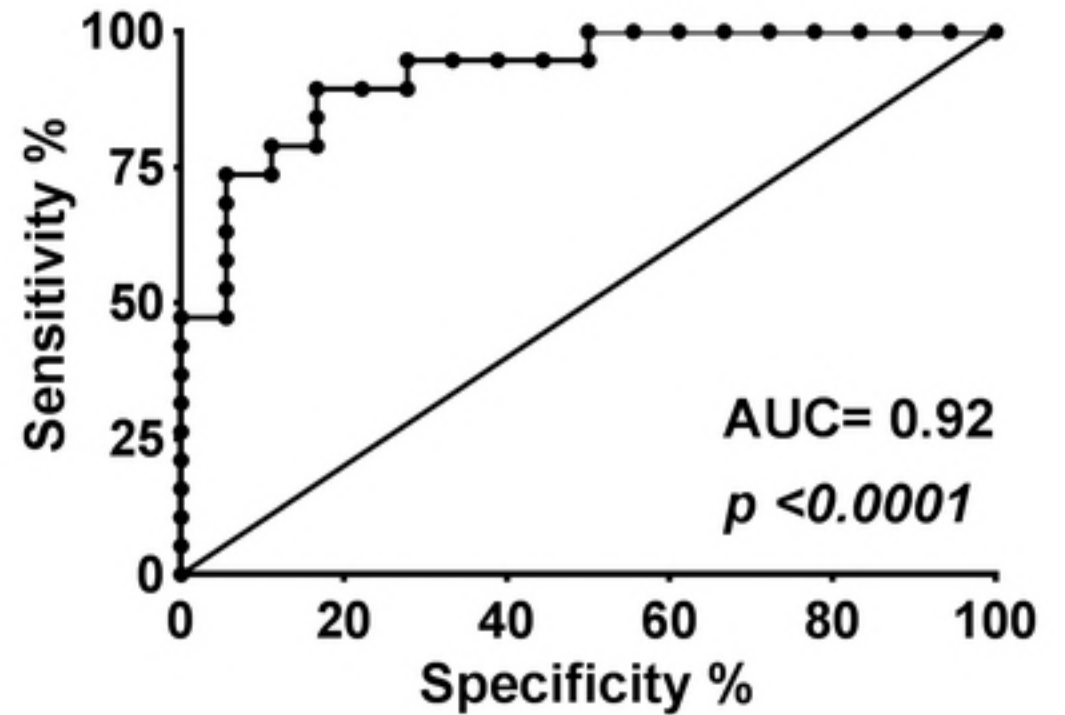
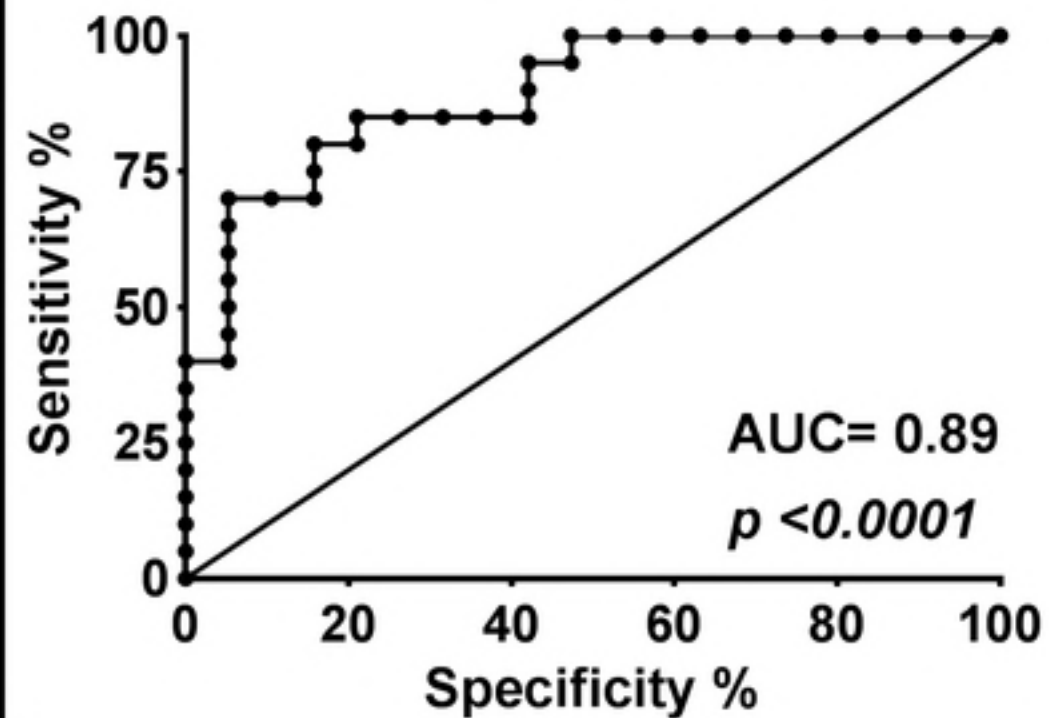
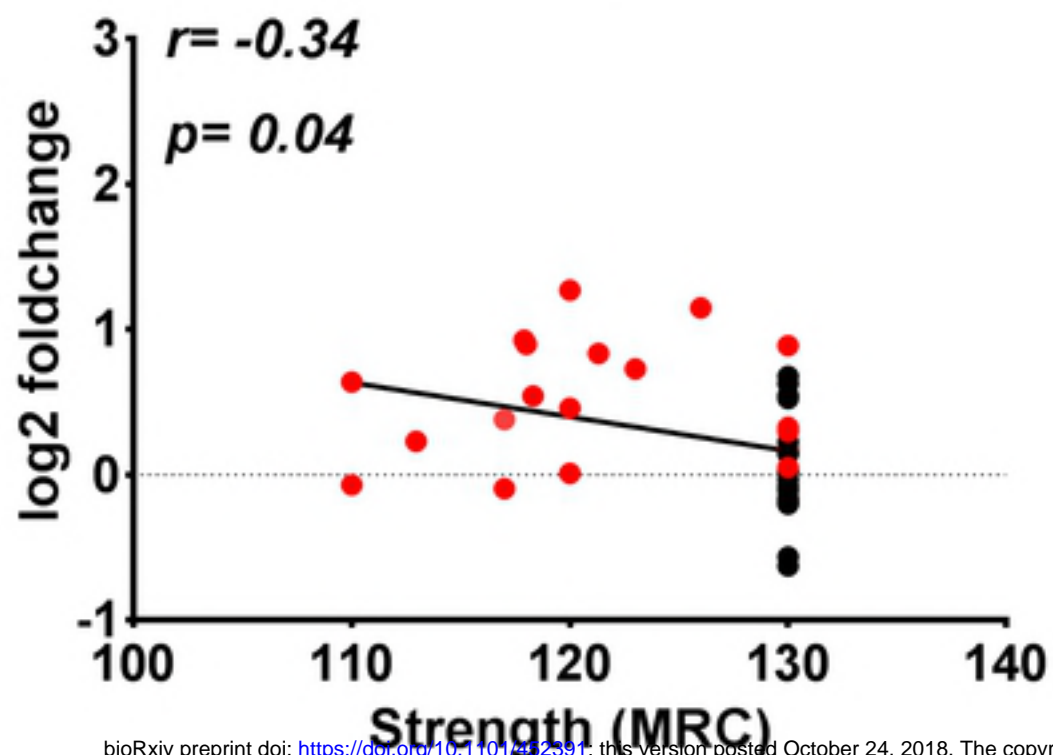
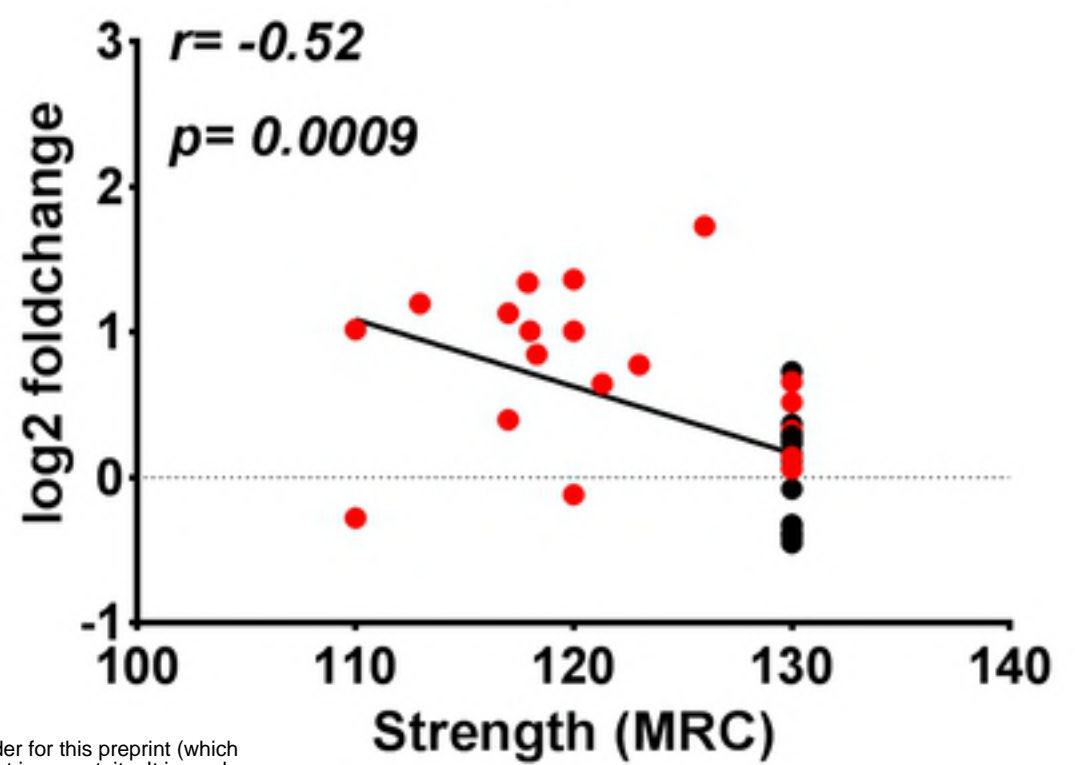
*circ/lin* RTN4\_03*circ/lin* ZNF609**b***circ/lin* score

Figure2

**a***circ/lin CDYL**circ/lin HIPK3*

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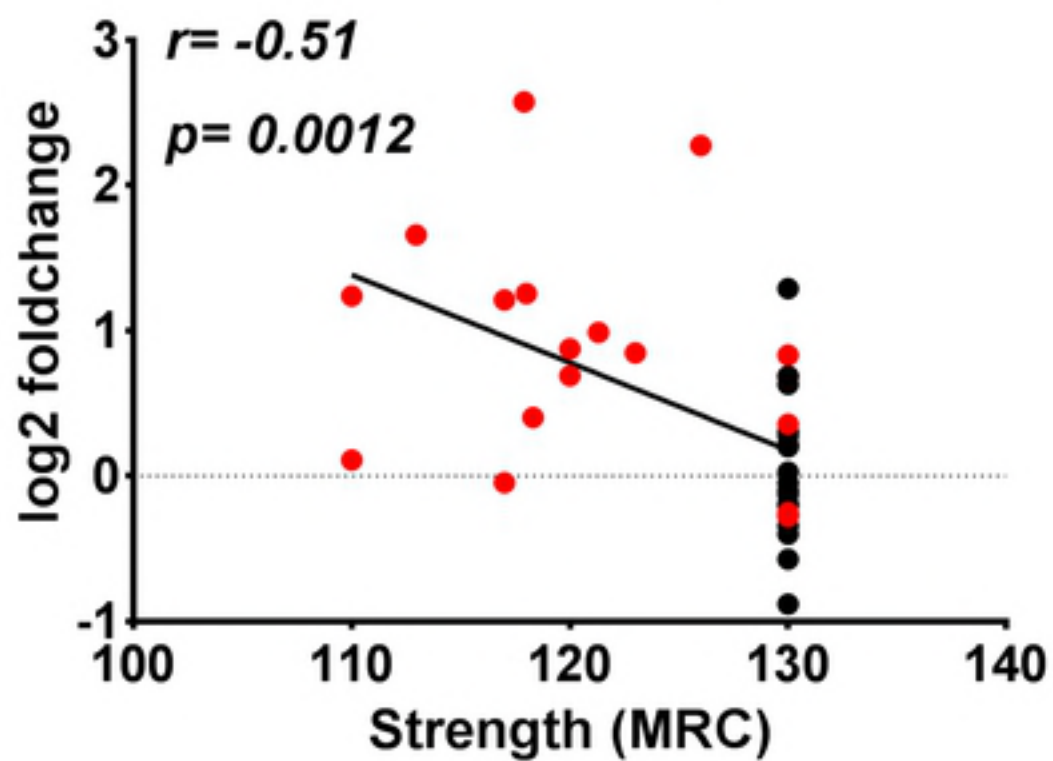
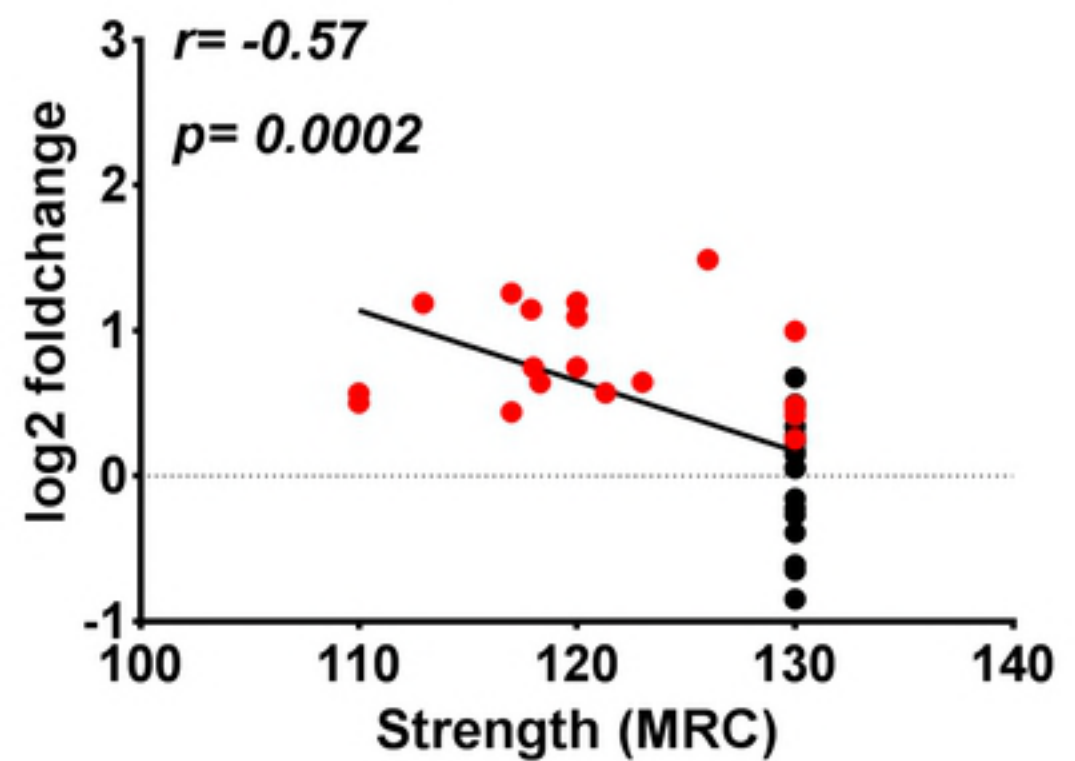
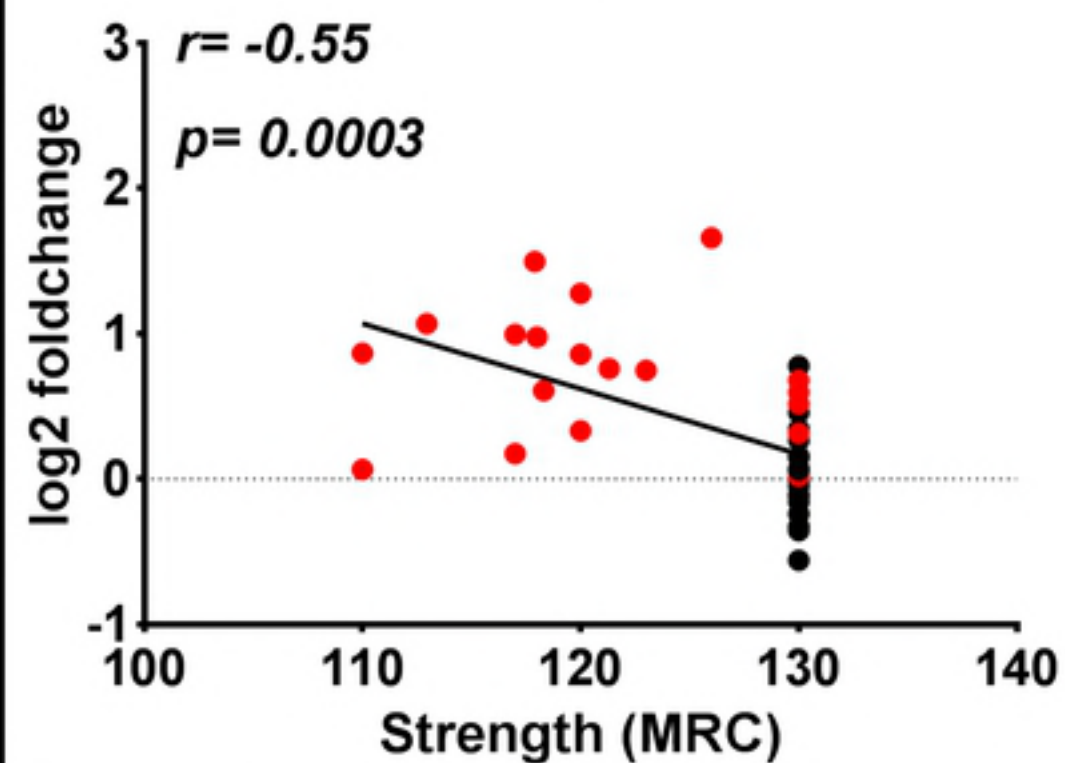
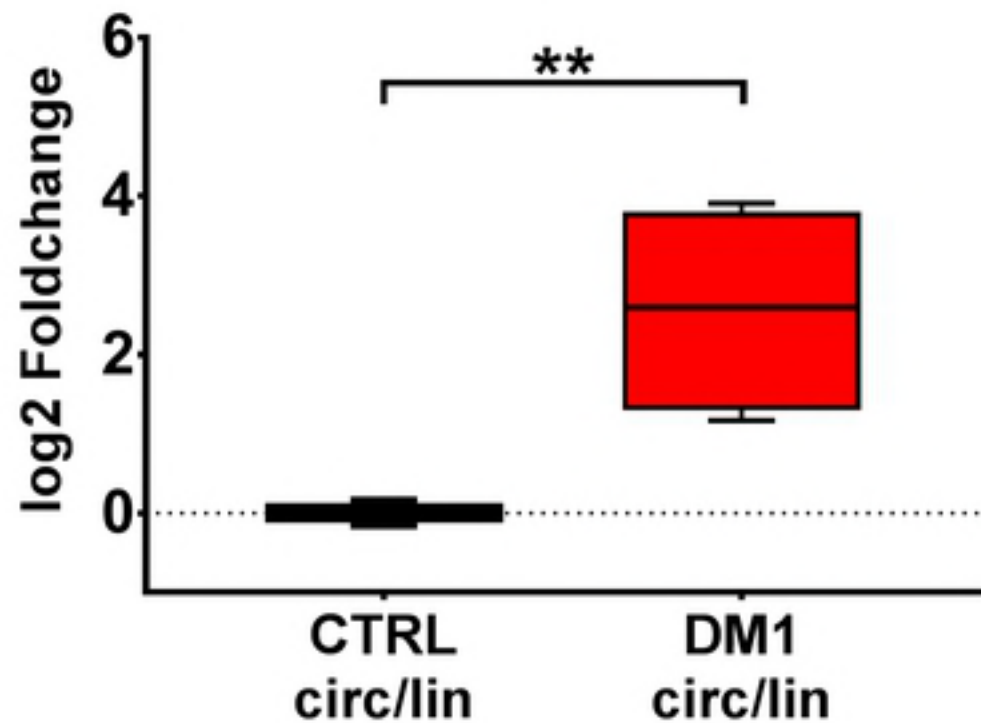
*circ/lin RTN4\_03**circ/lin ZNF609***b***circ/lin score*

Figure3

### RTN4\_03



### ZNF609

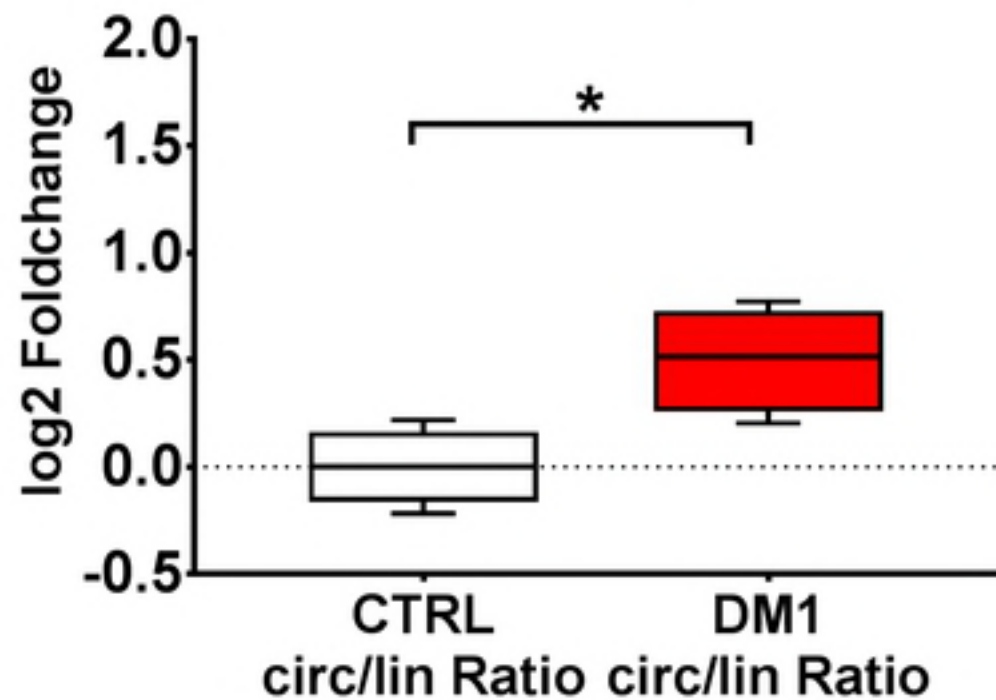
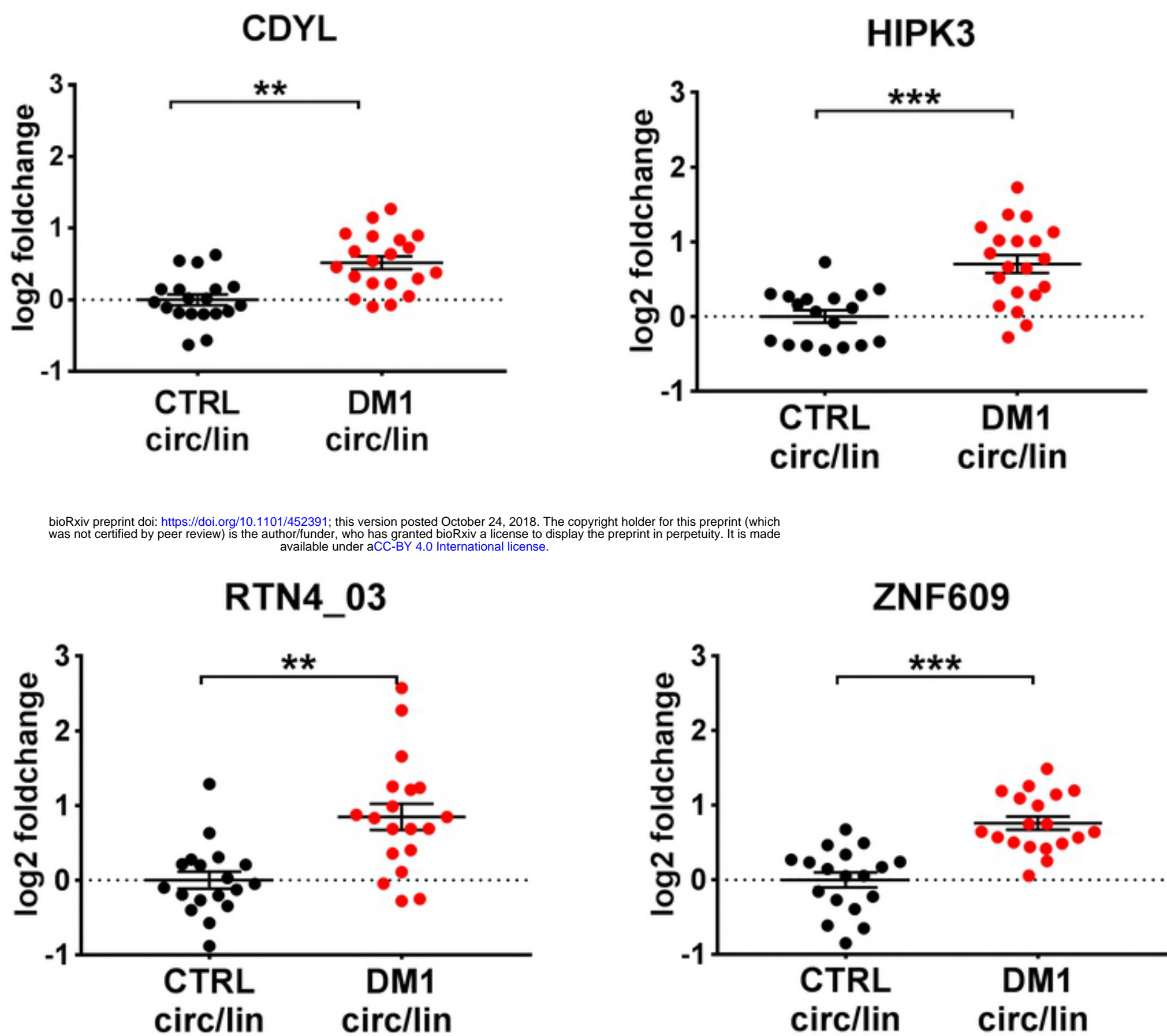


Figure4



**a**

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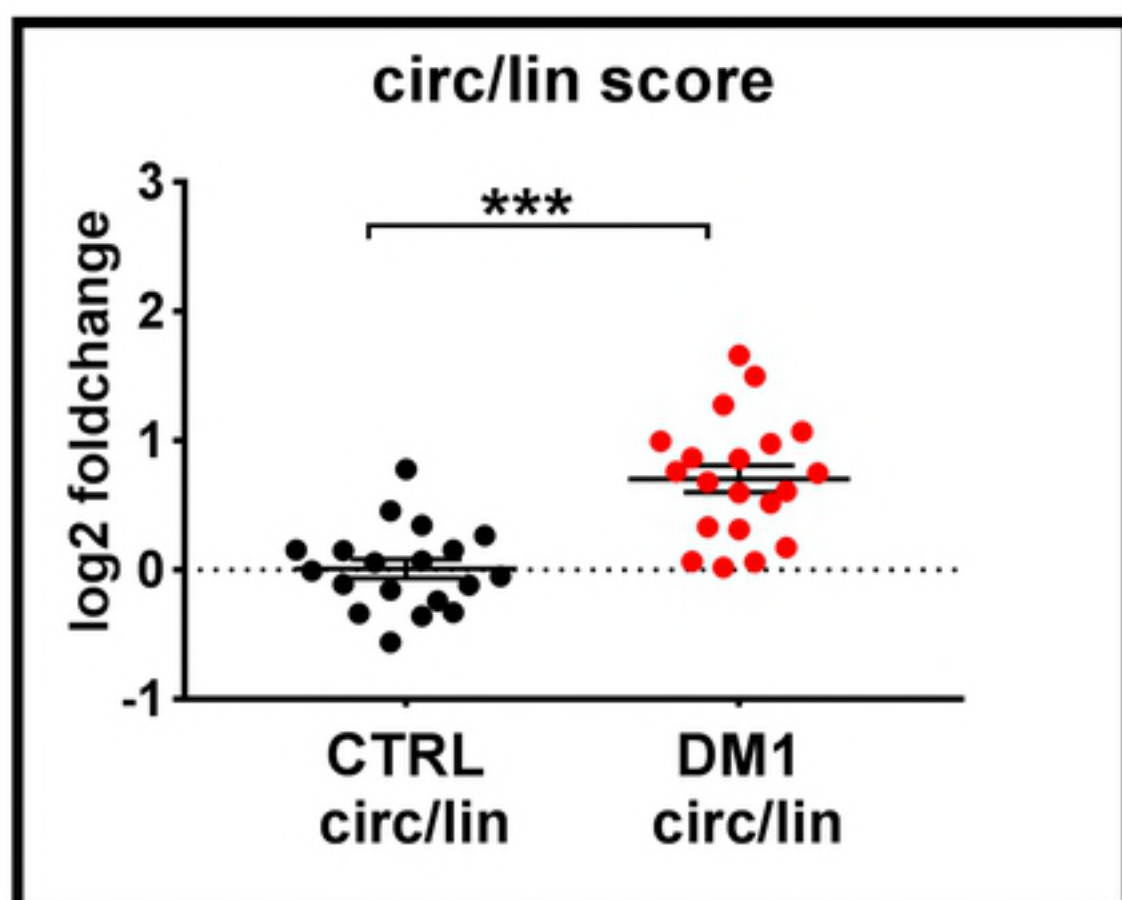
**b**

Figure 1