Novel Drosophila model of myotonic dystrophy type 1: phenotypic characterization and genome-wide view of altered gene expression

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Received February 11, 2013; Revised and Accepted March 13, 2013

Myotonic dystrophy type 1 (DM1) is a multisystemic RNA-dominant disorder characterized by myotonia and muscle degeneration. In DM1 patients, the mutant DMPK transcripts containing expanded CUG repeats form nuclear foci and sequester the Muscleblind-like 1 splicing factor, resulting in mis-splicing of its targets. However, several pathological defects observed in DM1 and their link with disease progression remain poorly understood. In an attempt to fill this gap, we generated inducible transgenic Drosophila lines with increasing number of CTG repeats. Targeting the expression of these repeats to the larval muscles recapitulated in a repeat-size-dependent manner the major DM1 symptoms such as muscle hypercontraction, splitting of muscle fibers, reduced fiber size or myoblast fusion defects. Comparative transcriptional profiling performed on the generated DM1 lines and on the muscleblind (mbl)-RNAi line revealed that nuclear accumulation of toxic CUG repeats can affect gene expression independently of splicing or Mbl sequestration. Also, in mblRNAi contexts, the largest portion of deregulated genes corresponded to single-transcript genes, revealing an unexpected impact of the indirect influence of mbl on gene expression. Among the single-transcript Mbl targets is Muscle protein 20 involved in myoblast fusion and causing the reduced number of nuclei in muscles of mblRNAi larvae. Finally, by combining in silico prediction of Mbl targets with mblRNAi microarray data, we found the calcium pump dSERCA as a Mbl splice target and show that the membrane dSERCA isoform is sufficient to rescue a DM1-induced hypercontraction phenotype in a Drosophila model.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a common neuromuscular disorder affecting 1 in 8000 people worldwide. DM1 patients display myotonia, muscle weakness and degeneration, together with insulin resistance, cataracts, cardiac conduction defects and hypogonadism.

This autosomal-dominant disease is caused by an unstable CTG triplet expansion in the 3′ untranslated region of the DMPK gene (1,2). The severity and age of onset of DM1 are correlated with the number of repeats (3). Briefly, normal individuals have 5–37 CTG repeats, patients with the adult or childhood form display from 50 to 1000 CTG repeats, and congenital DM1 patients can have several thousand triplet repeats.

Despite the complexity of DM1 pathogenesis, it is now well established that non-coding CUG repeat transcripts play a toxic gain-of-function role. Abnormal DMPK transcripts form secondary structures (4), which aggregate into foci within muscle nuclei (5,6) and which sequester RNA-binding proteins such as Muscleblind-like 1 (MBNL1) (7). Also, by a still undetermined mechanism activating protein kinase C, CUG-binding protein 1 (CUGBP1) is stabilized by phosphorylation (8). MBNL1 and CUGBP1 are both splicing factors, but play antagonistic roles (9). Thus, in DM1 patients, several transcripts are mis-spliced due to an inverse ratio of MBNL1/CUGBP1. Among mis-spliced transcripts, insulin receptor (IR) (10), chloride channel-1 (ClC-1) (11), Bin1 (12) and troponin T (cTNT) (13) are involved in insulin resistance, myotonia, muscle weakness and reduced myocardial
function observed in patients. However, recent reports (14,15) indicate that other molecular aberrations such as altered maturation of miRNAs or CUG repeat-dependent transcription factors leaching can also contribute to the pathogenesis of DM1. To characterize molecular defects underlying this pathology, several animal models have been generated. The first to be developed were mice models (8,16,17), but it was found that Drosophila also represented an accurate model system to study DM1 (18,19). Accordingly, fruit flies expressing CUG repeats in adult muscles develop DM1 symptoms (18,20) and were used to screen modifiers of transcript toxicity (21,22). Recently, applying a Drosophila model has revealed the role of the anti-sense DMPK transcript in DM1 pathogenesis (19). It has also been shown that Muscleblind (Mbl), the Drosophila MBNL1 ortholog, as in humans, is involved in muscle phenotypes observed in DM1 flies (20,21).

In this study, we report on the generation and phenotypic characterization of a novel Drosophila DM1 model composed of inducible transgenic lines carrying increasing numbers of CTG repeats inserted into the same genomic locus. To date, it is the only DM1 model which allows assessing effects of CTG repeats size. We show that by targeting CTG repeat expression or Mbl attenuation to embryonic and larval muscles, the DM1-like muscle defects can be detected as early as in third instar larvae. We also observe that the number of nuclear foci in muscle nuclei depends on the repeat size and that these foci sequester Mbl. As observed in patients (23,24), DM1 larvae display altered muscle morphology with splitting fibers and affected muscle function manifested by hypercontraction and impaired motility. We also demonstrate that under pathological conditions, larval muscles have a reduced number of nuclei due to affected myoblast fusion.

In an attempt to get further insights into DM1 pathogenesis, we used our model for comparative genome-wide analyses of gene expression and provide a large-scale view of repeat-dependent versus Mbl-dependent gene deregulation. The transcriptomic approach has been complemented by an in silico prediction of Mbl targets. These analyses revealed a set of genes whose deregulation appears Mbl-independent and more generally splice-independent. Among candidates whose deregulation is splice-independent, we found that the reduced transcript levels of Muscle protein 20 (Mp20) gene were involved in myoblast fusion defects observed in DM1 larva. We also found dSERCA transcript as a target of Mbl and showed that the mis-splicing of dSERCA transcripts leads to a decrease of its transmembrane isoform, resulting in altered muscle contractility.

RESULTS

Expanded CUG repeats accumulate in nuclear foci and sequester Mbl in a new Drosophila larval model of DM1

In order to better understand the effect of the repeat size on muscle parameters in DM1 patients, we generated a novel Drosophila model of DM1. Four inducible (UAS-iCTG) transgenic Drosophila lines carrying 240, 480, 600 and 960 interrupted CTG repeats were created. The link between these interruptions and DM1 symptoms remains unclear, but one could consider a possibility of additive effect in all so far generated animal models carrying interrupted CTG repeats including our model. To avoid effects of insertion sites on transgene expression, we applied a phage C31 integrase-driven site-specific transgenesis system (25,26). In previously described Drosophila DM1 models, only adult muscles and in particular the indirect flight muscles have been targeted and analyzed. However, there are two waves of myogenesis during the Drosophila life cycle. The first wave leads to the formation of larval musculature. The second wave, occurring during metamorphosis, gives rise to the adult fly muscles. We reasoned that targeting muscle cells from the early developmental stages with increasing number of repetitions would result in affecting larval muscles. To test this hypothesis, we first analyzed larval muscles for nuclear foci formation which are associated with DM1 pathogenesis (5,6). When crossed to a muscle-specific Mef-GAL4 driver, the UAS-iCTG repeat lines led to the synthesis of a non-coding RNA expressed at the same level in larval somatic muscles in all DM1 lines (Fig. 1A). Nevertheless, the longer the repeats are, the more foci we could detect in larval muscle nuclei. No foci could be observed in the DM1240 line, only few foci were detected in DM1480 (not shown) or DM1600 lines, whereas numerous foci were present in the DM1960 line (Fig. 1B). Since in a given line all muscle nuclei displayed a similar pattern of foci, we estimate that the Mef-GAL4-induced pan-muscular expression of the iCTG transgenes was relatively homogenous. Thus, the presence and the repeat-size-dependent abundance of nuclear foci suggest that larval muscleaturace can be suitable for analyzing DM1 phenotypes.

It has been shown in DM1 patients (8) and in DM1 model organisms (15,16) that the foci forming repeats sequester MBNL1/Mbl factor involved in splicing. We thus tested whether in our larval Drosophila DM1 model the Mbl protein also associates with foci. We found that Mbl co-localizes with foci in muscle nuclei (Fig. 1C), suggesting that in lines expressing a high number of CUG repeats the level of functional Mbl protein is strongly reduced. To mimic the Drosophila model the decreased availability of MBNL1 observed in DM1 patients we used a UAS-mblRNAi line allowing targeted attenuation of mbl gene expression. The validity of this line in the larval model was assessed, respectively, by qRT–PCR (Fig. 1D) and by immunodetection (Fig. 1E), showing that after muscle-specific attenuation, the overall mbl expression fell to ~50% (Fig. 1D) and that the Mbl protein in larval muscle nuclei was barely detectable (Fig. 1E). Accordingly, the muscle-targeted attenuation of mbl was subsequently used as a positive control condition.

Embryonic and larval lethality of pathological lines was assessed. We found that the expression of 960 CTG repeats in muscles was lethal in ~30% of embryos (Fig. 1F), but shorter non-coding CUG transcripts did not affect embryonic viability. It is noteworthy that the Mef>mblRNAi line displayed a moderate embryonic lethality (~17%), increasing to >30% in larval stages (Fig. 1F).

Reduced motility and affected muscle morphology of DM1 larvae

Third instar Drosophila larvae have well-developed locomotor behavior and are well-suited for assessing muscle performance using behavioral tests. Here, we used a contraction assay
(Fig. 2A) and a righting assay (Fig. 2B) to test the capacity of larvae to perform ordinary and complex movements, respectively (27). For the contraction assay, the number of peristaltic contractions performed by the larva within 30 s was recorded. An average of one contraction per second was performed in control lines, whereas under pathological conditions this number was significantly reduced. However, the pathological line with a large number of repeats (Mef >240CTG) and the mbl RNAi line, both performed slightly better than milder pathological lines. This can be explained by less extensive contractions in these lines, which could compensate for the number of contractions. For the righting assay, we put the larva in a dorsal position and recorded the time needed to revert to the crawling position. Control lines needed between 2 and 5 s, whereas the mbl attenuated line and the different DM1 lines needed a significantly longer time ranging from 10 s in the case of 240 CTG repeats to 20 s in the case of 960 CTG repeats (Fig. 2B). Thus, motility was affected in these lines. It is noteworthy that motility was also affected in the line Mef >240CTG which did not display detectable foci. In order to understand why motility is impaired, we analyzed the morphology of muscles and their pattern in mutant lines. We found several morphological abnormalities in body wall musculature of Mef >960CTG and DM1 larvae such as muscle splitting as well as loss of muscle fibers or presence of extra fibers (Fig. 2C). The average number of defects observed was significantly increased in pathological lines compared with the control line. Interestingly, the greater the number of repeats in DM1 lines, the more morphological defects we observed (Fig. 2D), a phenotype that correlates with the affected motility (Fig. 2B). The only exception was the DM1960 line, which displayed a significantly increased rate of splitting muscles compared with other DM1 lines, but a lower rate of extra fibers (Fig. 2D). We hypothesized that extra fibers could be a consequence of splitting that the affected muscles underwent from one end to the other. As we did not detect any extra fibers in DM1 embryos (Fig. 2D), extra fibers do not form by abnormal specification,
but rather result from the affected integrity of growing muscles, leading to their fragmentation during larval stages. In the case of the DM1₉₀₀ line, which exhibited relatively high lethality during the late embryonic stage (Fig. 1E), a reduced rate of extra fibers observed may be due to the fact that individuals with increased muscle fragmentation do not survive until the first larval instar.

It is well known that in cell culture, the fusion ability of DM1 myoblasts or satellite cells is altered (28,29), impacting on the myotube size. We thus examined in our larval Drosophila DM1 model whether the muscle size and the number of nuclei per muscle were affected. As shown in Figure 3A, the size of relaxed VL3 fibers was significantly decreased in DM1₆₀₀ and DM1₉₀₀ lines and in the line attenuated for mbl compared with their respective controls. Besides that, the number of nuclei per muscle was significantly reduced (Fig. 3B and C), indicating that the fusion process was affected in these pathological lines and that Mbl was involved in regulating myoblast fusion. The same observation was made in SBM fibers where fusion was also affected in all these lines (Supplementary Material, Fig. S1B and C). As the fusion process takes place in embryonic and not in larval stages, we conclude that the defects observed in larval muscles result in part from the deleterious effects of CTG repeats on

Figure 2. Altered motility in DM1 larvae is associated with an affected muscle pattern including splitting fibers. (A and B) Third instar larvae behavioral assays. (A) DM1 larvae display a crawling phenotype compared with controls (driver control and transgenic control). The graph shows the average number of larval peristaltic contractions/30 s recorded for each genotype. (B) DM1 larvae and Mef > mblRNAi line exhibit difficulty in performing complex movements compared with controls (driver control and transgenic control). The graph depicts the average time recorded for the larvae of each genotype to turn over. (C and D) Assessment of the overall muscle pattern and muscle abnormality quantification. (C) Images of in vivo scanning of the global muscle pattern carried out on third instar larvae segments A5–A7. Arrows point to splitting fibers. White arrowheads indicate extra fibers, brown arrowheads indicate missing fibers. (D) Table recapitulating the frequency of each defect observed in vivo for each mutant line but also the frequency of extra fibers in late embryos. *P < 0.05, **P < 0.01, ***P < 0.001 versus Mef > lacZ line.
muscles during embryonic development. We also noted that in muscles of DM1 larvae, the distribution of nuclei along the fiber was not affected (Fig. 3C and Supplementary Material, Fig. S1C).

Impaired muscle relaxation in a Drosophila larval model of DM1

Myotonia is the characteristic DM1 symptom observed in patients. It leads to hypercontracted muscles, which appear shorter. Interestingly, attenuation of mbl via RNAi or muscle-targeted CTG repeat expression in Drosophila larvae results in a significantly reduced length of muscles (Fig. 3A). This may also be due to either a reduced number of sarcomeres or a decreased sarcomere size. To distinguish between these two possibilities, we counted the sarcomeres and measured their size in relaxed fibers fixed in the presence of EDTA, a calcium chelator. The profile of Z-line staining with phalloidin revealed that the number of sarcomeres in VL3 fibers was similar in control lines and in DM1 lines (Fig. 3D), whereas the line attenuated for mbl and the DM1 lines expressing 600 and 960 CTG repeats displayed progressively significant decreased sarcomere size (Fig. 4A and B).

The reduced sarcomere size observed in DM1 larval muscles relaxed with EDTA suggested that we could observe the hypercontracted phenotype in our model at the fiber scale. To confirm this possibility, we measured the contractility index of each line by comparing the size of fibers relaxed with EDTA with the size of contracted fibers. We observed that the contractility index was strongly affected in the line attenuated for mbl and in the DM1960 line (Fig. 4C). We expected that in these two genetic contexts, fibers would not undergo efficient relaxation when treated with EDTA and thus display a particularly low contractility index. Several channels already shown to be mis-spliced in the disease, such as the chloride channel 1 (11) and possibly the Ca pump SERCA (30) could be responsible for this phenotype.

Transcriptional profiling of DM1 CTG-repeat lines and mblRNAi line reveals splice-independent gene deregulation

DM1-like phenotypes observed in larval muscles of generated Drosophila lines prompted us to use them for transcriptional profiling in an attempt to gain insight into unknown facets of DM1 pathogenesis. One important question that remained to be addressed was the contribution of splice-independent gene deregulation to the global gene expression defects in DM1.

To approach this issue, we performed microarray analyses on the following three conditions: Mef>mblRNAi, Mef>600CTG and Mef>960CTG versus the Mef>lacZ control condition. We found that among all up- and down-regulated
genes (Supplementary Material, Table S1) in Mef > 600CTG and Mef > 960CTG contexts, those whose expression changed in CTG repeat lines but not in the Mef > mblRNAi line (Fig. 5A) represented \( \approx 35\% \) (291 genes out of 833 with at least 2-fold up- or down-regulation).

Within this pool of CTG-only deregulated genes, most (69.5%) are known to produce only one transcript (Flybase, http://flybase.org/), suggesting the possibility that the mechanism other than splicing contributes to their regulation. Thus, our data show that Mbl-independent and more importantly, splice-independent gene deregulation can be detected in muscles expressing toxic repeats. Among candidates identified as specifically down-regulated by CTG repeats and described as producing only one transcript are genes involved in metabolic processes, in particular in carbohydrate metabolism and oxidation-reduction processes (Fig. 5B). Five genes from each category stated above were selected for validation (Amy-d, Amy-p, CG32444, CG9466 and CG9468 for the metabolism group and Cyp6a18, v, Cyp6w1, Cyp304a1 and CG2065 and an up-regulation for Cyp6a18 (Fig. 5C and D). We also found that Amy-p, Cyp304a1 and CG2065 were actually down-regulated to a lesser extent in Mef > mblRNAi larvae, highlighting higher sensitivity of RT-qPCR compared with microarrays, and revealing a potential role of Mbl in indirectly regulating transcriptional activity of genes. We also observed that CG9466 and CG9468 down-regulations were Mbl-dependent.

A global view of repeat-size-dependent gene deregulation

Interestingly, in the validation experiments presented above, we observed that expression of Amy-d, CG32444, CG9466, CG9468 and Cyp6w1 was more strongly affected in the DM1960 line than in the DM1600 line (Fig. 5E and F). We assigned misregulated candidates to three groups: DM1600-specific genes, DM1960-specific genes and common DM1 genes. We note (i) that more genes were deregulated in the DM1960 condition than in the DM1600 line (Fig. 5E) and (ii) that they were most...
Figure 5. A global view of splice-independent and repeat-size-dependent gene deregulation in a Drosophila larval model of DM1. (A) Venn diagram showing transcript distribution between Mef>mbIRNAi context and DM1 conditions. The diagram was generated from lists of transcripts that are >2.0-fold enriched or depleted, relative to the Mef>lacZ reference. For each compartment, the colored area represents the enrichment in single transcripts. (B) Pie chart recapitulating the Gene Ontology based biological process distribution of CTG-dependent and splice-independent mis-expressed genes on microarrays. Genes with an unknown biological process and molecular function were not taken into account in this chart (n = 95). (C and D) Validation of some candidate genes by RT–qPCR among CTG-dependent and splice-independent genes. (C) Amy-d, Amy-p, CG9466 and CG9468 belong to the carbohydrate metabolism group, whereas CG32444 is involved in nitrogen metabolism. All are down-regulated in microarray data. (D) Cyp6a18, v, Cyp6w1, Cyp304a1, CG2065 are genes involved in oxidation–reduction processes. All are down-regulated on microarray data apart from Cyp6a18 which is up-regulated. (E) Venn diagram showing transcript distribution between Mef>600CTG larvae (DM1600) and Mef>960CTG larvae (DM1960). The diagrams are generated from the lists of transcripts that were >2.3-fold enriched or depleted, relative to the Mef>lacZ reference. (F) Classification of repeat-size-dependent deregulated genes according to Gene Ontology. For each sub-group (DM1960 specific, DM1600 specific and common DM1 genes), classification takes into account up- and down-regulated genes. Genes with an unknown biological process and molecular function were not taken into account (DM1960 specific n = 92; DM1600 specific n = 51; common DM1 n = 58). The category ‘Other biological processes’ is enriched in genes involved in the structural constituent of chitin-based cuticle in DM1960 context most probably because of affected mobility and delayed emergence from medium of these third instar larvae. (G) Validation of some repeat-size-sensitive candidate genes by RT–qPCR. #P < 0.05, ##P < 0.01, ###P < 0.001 indicate significant difference in data distribution between genotypes (Kruskal–Wallis ANOVA test). ∗P < 0.05, ∗∗P < 0.01 indicates significant difference versus Mef>lacZ (Mann–Whitney test). Bars indicate difference between Mef>600CTG versus Mef>960CTG conditions (Mann–Whitney test).
often down-regulated (Fig. 5F). Among the GO classes that were over-represented when classifying DM1<sub>960</sub> specific down-regulated genes, we found the ‘Transport’ class (red in Fig. 5F). We thus reasoned that by classifying candidates from the over-represented GO classes based on the ratio of their fold-change between Mef<sub>2</sub>:<sup>600CTG</sup> and Mef<sub>2</sub>:<sup>960CTG</sup> conditions, we should be able to rank repeat size-sensitive genes. In this ranking we prioritized candidates that had human orthologs, and we set a threshold of fold-change ratio at 1.8, sorting out 26 down-regulated genes and 17 up-regulated genes (Supplementary Material, Table S3b). As genes belonging to the ‘Transport’ GO category were over-represented among candidates down-regulated specifically in the DM1<sub>960</sub> condition, we picked two high-ranked and evolutionarily conserved genes (Supplementary Material, Table S3b) from this category for validation: smvt (<i>sodium-dependant multivitamin transporter</i>) and CG17597 [ortholog of the human Sterol Carrier Protein-2 (SCP-2) gene]. RT-qPCR confirmed that both were down-regulated in a CTG repeat-size-dependent manner. However, for both of them we observed slightly reduced expression also in the DM1<sub>600</sub> context (Fig. 5G). We then extended our analyses to two other highly ranked genes: Myosin Heavy Chain (<i>Mhc</i>) and <i>Mp20</i> (orthologs are already known to be affected in DM1 (13,31,32). Mhc and <i>Mp20</i> code for major components of sarcomeres and as shown in Fig. 5G are down-regulated specifically in the DM1<sub>960</sub> condition. Thus, this analysis identifies a set of genes whose deregulation is repeat-size-sensitive and can represent potential markers of disease progression.

### Mp20, an indirect Mbl target involved in DM1-associated myoblast fusion defects

Among genes whose expression was altered in Mbl-only and Mbl/CTG repeat contexts were single-transcript genes (Fig. 5A and Supplementary Material, Table S4), indicating that Mbl can affect gene expression in a splice-independent way, most probably indirectly via mis-splicing transcription factors. Gene family distribution of both CTG and Mbl-dependent candidates, which correspond to single-transcript genes (Fig. 6A), shows that proteolysis and lipid metabolic processes are mainly affected. However, a candidate from a different category encoding an actin interacting protein caught our attention: <i>Muscle protein 20</i> (<i>Mp20</i>).

<i>Mp20</i> is known to be a positive modulator of myoblast fusion (33) during embryonic myogenesis. To validate decreased expression of <i>Mp20</i> detected by microarrays, we first performed a series of RT-qPCR experiments. We found that <i>Mp20</i> expression was indeed reduced in Mef<sub>2</sub>:<sup>mbiRNAi</sup>, Mef<sub>2</sub>:<sup>600CTG</sup> and in Mef<sub>2</sub>:<sup>960CTG</sup> larvae but also in late embryos when the myoblast fusion process takes place (Fig. 6B). This finding prompted us to test whether this Mbl-dependent transcriptional down-regulation of <i>Mp20</i> contributed to the affected myoblast fusion resulting in a reduced number of nuclei in larval muscles. We compensated for the reduced transcript levels of <i>Mp20</i> by targeting <i>Mp20</i> transgene expression to embryonic and larval muscles of <i>mbiRNAi</i> and DM1 lines. The experiment performed showed that <i>Mp20</i> was able to rescue DM1-associated fusion phenotypes (Fig. 6C and D), reestablishing wild type-like numbers of nuclei.

### In silico prediction of Mbl targets and microarrays identify transmembrane isofrom of the Drosophila sarco endoplasmic reticulum calcium-ATPase (dSERCA) as required for proper muscle contraction in DM1

To further investigate the involvement of Mbl in DM1, we sought to predict its mRNA targets using the YGCU(Y)nGCY consensus motif generated from sequences reported as bound by Mbl (34–36). We then searched for this motif in all intronic sequences of <i>Drosophila</i> genes referenced as alternatively spliced in the Flybase database and having a human ortholog. This led us to identify 1314 genes that could potentially be targeted by Mbl (Supplementary Material, Table S5). This pool of <i>in silico</i> Mbl target genes producing transcripts that undergo alternative splicing was compared with splicing-dependent genes whose transcript level is altered in Mef<sub>2</sub>:<sup>mbiRNAi</sup> larvae. In all, 112 candidates were sorted out as common <i>in silico</i>/microarray Mbl targets (Fig. 7A, Supplementary Material, Table S6), among them (Fig. 7B) the pump and transporter category.

As shown previously, both DM1 lines and the <i>mbi</i> attenuated line exhibited altered contractility (Fig. 4C), which could be due to a transcriptional deregulation or mis-splicing of calcium channels and/or chloride channels. Interestingly, among the common <i>in silico</i>/microarray Mbl targets identified we found dSERCA encoding a calcium pump. Since <i>in silico</i> prediction sorted out dSERCA as a target of the Mbl splicing factor and microarray data revealed differential expression of only a subset of dSERCA probes, it seems likely that dSERCA transcripts could be mis-spliced in DM1 muscles as already observed for SERCA1 in patients’ muscles (30). More precisely, the position of differentially expressed microarray probes suggests that the isoforms containing exon 8 or 11, encoding a sequence for the putative transmembrane domain (according to prosite scan software), could be down-regulated. In order to confirm that dSERCA was mis-spliced, we performed an RT-qPCR with a set of primers designed to specifically recognize the exons (8 or 11) coding for the transmembrane domain, and another set of primers designed to target the exons specific to the isoform A (exons 13 and 14) which does not contain this putative transmembrane domain (Fig. 7C). In normal conditions, we found that the transmembrane isofrom was predominantly expressed (Fig. 7D). However, under pathological conditions, we observed a decreased expression of transcripts containing the transmembrane domain and an increased expression of transcripts coding for the isoform A (Fig. 7D). dSERCA expression in larval muscles was assessed by immunostaining and showed reduced or absent dSERCA expression at muscle surface in the DM1<sub>960</sub> line and <i>mbi</i> attenuated line (Fig. 7E), but also in sarcomere for Mef<sub>2</sub>:<sup>mbiRNAi</sup> larvae in which dSERCA expression was more strongly affected. In addition, DM1 lines exhibited ectopic expression of dSERCA in nuclei. Thus dSERCA was mis-spliced in DM1 lines as well as in line attenuated for <i>mbi</i>, indicating that Mbl contributes to the mis-splicing. The accumulation in the nuclei of dSERCA in these lines (Fig. 7E) may correspond to an accumulation of dSERCA-A proteins.
As previously mentioned, dSERCA could be involved in DM1 hypercontracted phenotype. In order to validate this hypothesis, we first tested the effect of cyclopiazonic acid (CPA), a specific SERCA inhibitor (37), on the contractility index of the control line and hypercontracted lines. The Mef\textsuperscript{lacZ} control line exhibits an altered contractility index in the presence of CPA (Fig. 8A), showing that dSERCA inhibition can affect the contractility index. In addition, already hypercontracted lines, Mef\textsuperscript{960CTG} and Mef\textsuperscript{mblRNAi}, did not display any additive effect of CPA on their altered contractility index, suggesting that dSERCA is indeed an effector of hypercontraction in these lines. In order to rescue hypercontraction in DM1 and mbl attenuated contexts, we generated an inducible line that overexpressed the dSERCA transmembrane isoform and crossed it with hypercontracted mutant lines. Immunostaining showed that dSERCA expression was re-established at the muscle surface in both contexts, making it difficult to discern sarcomeres (Fig. 8B). Also, the contractility index was totally rescued in both Mef\textsuperscript{960CTG}, dSERCA\textsubscript{mb} and Mef\textsuperscript{mblRNAi},dSERCA\textsubscript{mb} lines (Fig. 8A).

**DISCUSSION**

*Drosophila* has already proved to be a powerful tool for conducting genetic screening and global analyses on the effect of CTG repeats in DM1 (21,22,38). So far, an inducible line expressing 480 interrupted CTG repeats has been used at the adult stage and shows age-dependent muscle degeneration (18). In this study, we generated a set of three inducible site-specific lines expressing 240, 600 or 960 interrupted CTG repeats. It has been observed in some cases of DM1 the existence of variant repeats interrupting the pure CTG expansions (39–41). Interestingly, interruptions allowed either repeat stabilization (39,40) or repeats contraction (41). If in one peculiar DM1 family CCG and CGG variants were associated with Charcot-Marie-Tooth symptoms (40), the role of interruptions remains unclear in other patients (41). The interrupted CTG repeats have already been used to generate different animal models of DM1 (21,42) including ours. One could consider a possibility of additive toxic effect in all these models. However, the CTCGA interruption motif commonly used for these models (21,42) is different from already described variants and its toxicity as well as unstability were not reported so far.

We used CTG size variation to compensate for age effect in third instar larvae. Here, larval muscles were studied instead of adult muscle for three reasons: (i) segmentally repeated larval musculature is organized in a stereotyped network of muscle fibers and is easy to analyze at morphological and functional levels, (ii) establishing and characterizing larval DM1 model appeared attractive for future genetic rescue approaches and molecular screening applications and (iii) adult lethality of...
the Mef > mblRNAi line prevented comparative analyses with DM1 lines in adult flies.

**Drosophila larval model recapitulates main DM1 muscle defects observed in patients and vertebrate models**

As observed in patients, we found that expressing an increasing number of CTG repeats in larval somatic muscles led to the formation of nuclear foci (5,6) and that these foci co-localized with Drosophila MBNL1 ortholog, Mbl (7). As the number of repeats positively influences the number of foci per nucleus and worsen muscle phenotypes, we consider that the new Drosophila model of DM1 presented here is of interest for simulating disease progression and (or) severity.

Global analysis of muscle pattern in our model revealed a histopathological defect called ‘splitting fibers’ already observed in mbnl1 knockout mice (43) as well as in DM1 patients (23,24). Here, splitting occurs during larval stages characterized by rapid muscle growth. As observed in dorsal oblique fibers, it is initiated at muscle endings at the level of interaction with tendon cells. This suggests that splitting results from affected muscle attachment to tendon cells and (or) abnormal sarcomeric organization that weaken the integrity of myofibrils. This latter hypothesis is supported by decreased expression of two sarcomere components (Mhc, up) in the DM1960 line. Surprisingly, the Mef > 240CTG line which did not exhibit visible foci within muscle nuclei displayed altered motility associated with muscle splitting but...
fusion defects in DM1, since by overexpressing the Mp20 binding protein involved in nuclei per fiber in our DM1 lines and in the during myogenesis we were able to rescue the number of line. Surprisingly, Mef0.05, **P, P, did not appear to undergo alternative splitting is suf-
fiber defects. This observation suggests that splitting is suf-
icient to alter motility.

Also, SBM and VL3 fiber examination shows reduced muscle size. So far, it has been shown in primary cell culture of myoblasts from DM1 patients that the ability of DM1-derived myoblasts to fuse is affected (29), consequently reducing myotube length. Here, we report for the first time that expressing non-coding CTG repeats affects in vivo myoblast fusion. Interestingly, microarray data and RT–qPCR performed at embryonic and larval stages on mutant lines have shown decreased expression of Mp20 encoding an actin-binding protein involved in Drosophila myoblast fusion (33). Mp20 appears as an attractive candidate gene for myoblast fusion defects in DM1, since by overexpressing Mp20 during myogenesis we were able to rescue the number of nuclei per fiber in our DM1 lines and in the mbl attenuated line. Surprisingly, Mp20 did not appear to undergo alternative splicing (single-transcript gene according to Flybase), suggesting that its Mbl-dependent down-regulation could occur through an indirect effect of Mbl. It is also noteworthy that one human counterpart of Mp20, the Calponin 3 gene, has recently been found involved in myoblast fusion in vitro (44). Thus, genes of the Mp20/Calponin family appear as attractive candidates to be tested for their role in DM1 muscle defects in humans.

Finally, we report here that mutant larvae and in particular those from DM1600 and Mef0.05, **P, P, lines. First, during validation of selected candidate genes from microarray analyses we identified repeat-size-dependent deregulation of genes involved in carbohydrate and nitrogen metabolism. A more systematic classification of candidates deregulated in a repeat-size-dependent manner and having human orthologs was then performed based on the ratio of their fold-change between the two conditions and on their function. Our data revealed that genes encoding transporter proteins were significantly enriched among gene categories down-regulated in larvae carrying high repeat numbers (DM1960 line).

Among these we validated repeat-size-dependent deregulation of smvt whose human orthologs (SLC5A3, SLC5A5, SLC5A8 and SLC5A12) encode myo-inositol transporters and CG17597/SCP-2 involved in phosphatidylinositol transfer and signaling (45). It is known that phosphatidylinositol is a derivative of myo-inositol, suggesting that both transporters may work in the same pathway. However, how the alterations of transporters influence the accumulation of the inositol forms and how this is connected to muscle defects observed in our DM1 model remain to be investigated.

We also found that two genes involved in the sarcomere structure Mhc and up were both down-regulated specifically in the DM1600 context. In DM1 patients, it was shown that Mhc ortholog MYH14 (31) and up orthologs TNNT2 and TNNT3 (13,32,43) were mis-spliced. Besides, a recent report provided evidence that in a Drosophila mbl null mutant, up transcripts are mis-spliced as well (46). However, the link between the Mhc and up gene deregulations and DM1 muscle phenotypes and their impact on DM1 pathogenesis have not yet been investigated. We speculate that down-regulation of Mhc and up might be involved in splitting fiber phenotype observed in DM1 larvae.

Genome-wide view of repeat-size-dependent gene deregulation in Drosophila DM1 model

As demonstrated here, the severity of several phenotypes is positively correlated with the size of the CTG repeats. This prompted us to carry out comparative transcriptional profiling on DM1600 and DM1960 lines. First, during validation of selected candidate genes from microarray analyses we identified repeat-size-dependent deregulation of genes involved in carbohydrate and nitrogen metabolism. A more systematic classification of candidates deregulated in a repeat-size-dependent manner and having human orthologs was then performed based on the ratio of their fold-change between the two conditions and on their function. Our data revealed that genes encoding transporter proteins were significantly enriched among gene categories down-regulated in larvae carrying high repeat numbers (DM1960 line).

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Comparative genomics of CTG repeat lines and mblRNAi line identify splice-independent gene deregulation in DM1

Comparative genomic analyses showed that a high percentage of genes with misregulated expression (~70%) did not undergo alternative splicing but were sorted out in the Mef. mblRNAi context. As Mbl binds specifically to double-stranded RNA structures (36), we hypothesize that it may influence transcript stability of this class of genes as already observed with MBNL1 in C2C12 cells (47). Alternatively, Mbl might play an indirect role on single-transcript genes via mis-splicing of transcription factors that regulate their expression. In order to gain insights into the second hypothesis, we tried to identify potential common regulators of Mbl-deregulated single-transcript genes using the bioinformatics i-cisTarget approach (http://med.kuleuven.be/lcb/cisTargetX2%22). Interestingly, several transcription factors known to act in muscles (Table 1) such as dMef2 and GATA factor Panier (Pnr) were found as potential transcriptional regulators of candidate genes. More importantly, the same transcription factors were found deregulated in transcriptional profiling experiments under all pathological conditions and most of them (including dMef2 and Pnr) were also predicted in silico to be targets of Mbl. Thus, these data reveal an important contribution of single-transcript gene deregulation in our Drosophila DM1 model and point to an indirect role of Mbl in the regulation of gene expression via mis-splicing of key myogenic factors. As a matter of fact, this mechanism may play a role in the regulation of Mp20 expression, one of dMef2 targets (Table 1).

<table>
<thead>
<tr>
<th>Transcription factors</th>
<th>Fold change Mef&gt;MefRNAi versus Mef&gt;lacZ</th>
<th>Mef&gt;600CTG versus Mef&gt;lacZ</th>
<th>Mef&gt;960CTG versus Mef&gt;lacZ</th>
<th>Total number of potential targets</th>
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<tr>
<td>Pnr</td>
<td>19.8006878</td>
<td>4.301325137</td>
<td>7.735258251</td>
<td>2 UP and DOWN, 53 UP, 161 DOWN</td>
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<tr>
<td>Grn</td>
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<td>dMef2</td>
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<td>0.89748296</td>
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<tr>
<td>ap</td>
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<td>1.808776213</td>
<td>1.892875924</td>
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<td>Antp</td>
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</table>

All the transcription factors were identified by analyzing single-transcript genes from the list of Mbl-dependent candidates using the i-cisTarget software (http://med.kuleuven.be/lcb/cisTargetX2%22). For each one, the fold change in the microarray experiment for different probes in each condition is shown as the number of putative targets.

*Indicates transcription factors sorted out by in silico prediction.

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expression is down-regulated in pathological contexts leading to myoblast fusion defects. Consequently, we suggest an indirect role of Mbl in Mp20 expression through misregulation of dMef2 alternative splicing.

**Combined in silico prediction of Mbl splice targets and transcriptional profiling of mblRNAi line reveal a key role of dSERCA in DM1-associated muscle contraction defect**

As discussed before, we observed in DM1_960 larvae an of dSERCA in DM1-associated muscle contraction defect transcriptional profiling of the transmembrane domain showed decreased expression in Mbl-deficient context, the exons 8 or 11 of dSERCA were spliced out, leading to the production of dSERCA isoforms devoid of the transmembrane domain. This switch in dSERCA isoforms is consistent with the immunostaining of DM1 larval muscles, in which the membrane-associated dSERCA protein was barely detectable at muscle surface or even in sarcomere for the Mef > mblRNAi line, whereas the level of free dSERCA in nuclei appeared to be enhanced in DM1 lines. It was previously shown that in DM1 patient muscles, as a result of MBNL1 sequestration, SERCA1 exon 22 in the 3’ part of the transcript is excluded (30,49) leading to the formation of a neonatal isoform of SERCA1. This isoform was expected to cause muscle degeneration, but so far, no functional analysis has been performed to confirm this hypothesis. However, patients with Brody’s disease, which is caused by different mutations in the SERCA1a gene, manifest impairment of skeletal muscle relaxation among other symptoms (50,51). In addition, it has been shown that dSERCA plays a key role in muscle contraction and heartbeat frequency and rhythmicity in flies (52,53), suggesting that it might be involved in muscle hypercontraction phenotypes and myotonia in DM1 muscles.

To date, the only gene functionally implicated in myotonia in DM1 is the **CIC-1** encoding a muscle-specific chloride channel. CIC-1 transcripts were found to undergo MBNL1- and CUGBP1-dependent splice modifications causing muscle delayed relaxation and pathogenic muscle defects (9,11). However, recent analyses performed on HSA(LR) myotonic mice have revealed that CIC-1 channels account for muscle hyperexcitability in young but not in old DM1 animals, suggesting alteration of conductance other than chloride currents (54). Thus, we decided to test whether the loss of dSERCA function and in particular depletion in its isoforms carrying the transmembrane domain could indeed affect muscle contractility. We first used a pharmacological tool, CPA, a highly specific inhibitor of SERCA (37), which binds to the entry channel (55) and found that the contractility of CPA-treated larval muscles is severely affected. We then performed rescue experiments by overexpressing the transmembrane isoform of dSERCA in DM1 lines with hypercontracted phenotypes and found that the contractility index was significantly improved. Thus, our data provide the first evidence in an animal model of DM1 that SERCA mis-splicing is involved in muscle hypercontraction.

In summary, we generated a new *Drosophila* model of DM1 and showed that Mbl-dependent mis-splicing of dSERCA caused muscle hypercontraction, the main skeletal muscle defect associated with DM1. Thus, this study supports the key role of MBNL1 sequestration in DM1 pathogenesis. On the other hand, our transcriptional profiling analyses suggest that mechanisms involving an indirect effect of Mbl account for gene deregulations and muscle phenotype in DM1. Among genes whose expression is indirectly regulated by Mbl, we identified Mp20/calponin and showed that it is able to rescue DM1-associated myoblast fusion defects *in vivo*. Our genome-wide data also reveal Mbl-independent and more generally splice-independent gene deregulation in pathological DM1 lines. This effect could be due to the sequestration of other factors by toxic repeats. Finally, we identified new candidate genes encoding sarcomeric proteins, myo-inositol transporters or involved in carbohydrate metabolism whose deregulation is repeat-size-dependent. These findings further support the complexity of DM1 and make our model a valuable tool for studying disease progression and (or) repeat-size-dependent severity.

**MATERIALS AND METHODS**

**Transgenic Drosophila line generation and Drosophila stocks**

All stocks were maintained at 25°C in standard medium in a 12 : 12 h light–dark cycling humidified incubator. UAS-iCTG240, UAS-iCTG 480 and UAS-iCTG 600 plasmids were generated following published procedures (56). UAS-CTG 660 was generated with repeats excised from a DMPK-CAG 806 plasmid (13,57). All these constructs were generated by concatenamerization of synthetic double-stranded DNA oligonucleotides of 20 CTG units interrupted by the sequence CTCGA. Site-specific transgenesis (performed by the Fly Facility platform, www.fly-facility.com) was then used to generate DM1 repeat lines. The site-specific M[vas-int.Dm]ZH-2A, M(3xP3-RFP.attP)ZH-86Fb (BL#24749, Bloomington) host line was used for injections (Fly Facility, Clermont-Ferrand, France). The UAS-Serca_mb rescue construct was subcloned from GH26644 (BDGP ESTs Collection) by amplification with high-fidelity DNA polymerase (Phusion, Biolabs), sequenced and cloned into the pUASAttb vector (26). The following pair of primers was used for amplification of Serca_mb sequence: 5'-atacgagcggtcgaagaggataacg-3’ 5'-atactcgagataaggtgctttag-3’. The site-specific PBac[y(+)-attP-3B]VK00037 (BL#9752) host line was used for injection (Fly facility, Clermont-Ferrand, France). w1118; P[GD13374]v28732 line referred to as UAS-mblRNAi (32) line was obtained from the Vienna Drosophila RNAi Center (VDRC, Vienna, Austria). The UAS-Mp20 rescue line was a gift from Bataillé et al. (33). The y[w1118] w[+]; P[w[+;+mC] = GAL4-Mef2.R]3 mentioned as Mef-Gal4 line was ordered from the Bloomington Stock Center (BL#27390). The Mef > mCD8GFP line was a kind gift from A. Paululat.
Lethality test

*Drosophila* crosses were maintained at 25°C on apple juice agar plates with yeast paste. The eggs laid were counted on the first laying day and the number of L1 larvae was counted on each subsequent day. To determine embryonic lethality, the following formula was applied: % embryonic lethality = 100 − [(L1 larvae)/(embryos)] × 100. No more than 25 L1 larvae were then transferred and grown on apple juice agar plates with yeast paste and the number of pupae was quantified. To determine larval lethality, the following formula was applied: % larval lethality = 100 − [(pupae)/(L1 larvae)] × 100.

**In situ** hybridization and immunofluorescence staining of *Drosophila* larval muscles

Third instar larva dissections were performed as previously described (58) in 0.9% NaCl buffer with 25 mM EDTA (except in the fiber contraction assay when EDTA was not added). Larval muscles were then fixed for 10 min in 4% formaldehyde on the plate, transferred to an Eppendorf tube, rinsed three times for 5 s in 1X PBS, 0.5% Tween (PBT), blocked for 20 min in 1X PBT, 20% horse serum at room temperature. Incubation with a primary antibody was performed for 2 h at room temperature with mouse monoclonal anti-Lamin C 28.26 (1:600; Hybridoma bank, Iowa, USA), rabbit anti-Mbl (1:1000; Proteogenix, Oberhausbergen, France) and rabbit anti-dSERCA (1:1000; (52)). Muscles were then washed three times for 10 min with 1X PBT and incubated with fluorescent secondary antibodies (1:300; Jackson Immuno-Research) for 1 h at room temperature and (or) with phallolidin-TRITC (1:1000; Sigma P1951) when appropriate. Larvae were mounted in Fluoromount-G anti-fade reagent (Southern Biotech) and were then analyzed using a FV300 (Olympus) confocal microscope.

**In situ** hybridization on larval muscles to detect foci was performed as previously described (20). Foci were imaged with a Leica SP5 confocal microscope.

**Muscle measurements**

Nuclei and sarcomere counting together with fiber length measurements with or without EDTA were carried out on VL3 (and SBM) fibers located in abdominal segments of larvae, under a ×20 objective. At most two segments were used for each larva and for each condition 18–22 measurements were performed. The fiber contractility index (FCI) was calculated as follows: FCI = (average size of relaxed fibers − average size of contracted fibers)/average size of relaxed fibers. The FCI measured in the presence of 25 μM of cyclopiazonic acid (CPA) was calculated as follows: FCI (+CPA) = (average size of relaxed fibers with CPA − average size of contracted fibers)/average size of relaxed fibers.

Sarcomere size measurements were performed as previously described (59) on six different VL3 muscles with three measurements of 100 microns per VL3 to homogenize sarcomere size along the fiber. The size of each sarcomere corresponds to the distance between two peaks on the plot profile generated by FIJI.

Motility tests and muscle pattern assessment

Motility tests are based on already published protocols (27). The contractility assay was performed by recording the number of peristaltic contractions executed by the larva during a 30 s move on a grape medium plate. The righting assay consists in putting the larva on its back and measuring the time needed for it to revert to its crawling position. A maximum of 60 s was recorded for larvae that failed to right themselves. Each test was performed alternatively on 10–11 larvae per genotype and repeated three times per larva.

In order to visualize a global muscle pattern, larvae expressing membrane GFP in muscles were sacrificed by heating at 70°C for 30 s. A lateral view of A5 to A7 abdominal segments was imaged on 20 larvae per genotype on a confocal microscope and splitting fibers, extra and missing fibers were quantified.

**RT–qPCR**

Total RNA was extracted from the whole third instar larva using a TRIzol reagent (Invitrogen) and the remaining DNA was removed using RQ1 DNase (Promega). Complementary DNA was synthesized from 5 μg RNA using a Superscript III First Strand Synthesis System for RT–PCR (Invitrogen). A PCR was performed using gapdh1 or Rp49 as controls and primers used are listed in Supplementary Material, Table S7. Quantitative RT–PCRs were performed in duplicate in a final volume of 20 μl on three to six different RNA extractions using a Power SYBR Green PCR Master Mix (Roche, Applied Science), on a LightCycler® 480 Real-Time PCR System (Roche, Applied Science). The following LightCycler experimental-run program was used: denaturing program (95°C for 10 min), amplification and quantification program repeated 40 times (95°C for 10 s, 58°C for 10 s, 72°C for 15 s). Data were analyzed using the ΔΔCt method. Owing to the small number of samples, we could not perform a parametric test since we were unable to verify data normal distribution. We opted for the non-parametric Kruskal–Wallis ANOVA test to compare all groups (i.e. Control, Mef > mblRNAi, Mef > 600CTG and Mef > 960CTG) in order to determine whether their differences of distribution were significant. When appropriate, we then focused on comparison between control samples and samples of interest using the non-parametric Mann–Whitney test, thus confirming significant difference.

**In silico prediction of Mbl targets**

The putative Mbl targeted YGCUYnGCY motif was screened in the FASTA Flybase intronic database (http://flybase.org/static_pages/docs/datafiles.html) using software developed for this purpose with the Perl CGI language. Transcripts were filtered and kept when they had both several possible isoforms and a human ortholog (Biomart, Ensembl). The position and number of the screened motifs were sorted out for each transcript. Genes coding for sorted transcripts were then classified depending on their expression profile (BDGP, http://www.fruitfly.org/cgi-bin/ex/insitu.pl) and the biological process they were involved in (Flybase).
Microarray analysis

Total RNA was extracted from whole third instar larvae using TRIzol reagent (Invitrogen). Three independent RNA isolations were performed for each of four genetic contexts. The Agilent Drosophila gene expression microarrays were used (Agilent, G2519F, Strasbourg, France). First, Treeview (version 1.60, University of California at Berkeley) was used to assess the similarity between triplicates. Only replicates with a 70% Pearson’s correlation were kept. Secondly, a $P < 0.001$ and a 2-fold increase/decrease were the thresholds to consider a probe differentially expressed. Differentially expressed genes were then classified according to the biological process they were involved in (Flybase).

Microarray data have been deposited in the ArrayExpress database with accession code E-MTAB-1469.

General statistical analysis

All statistical analyses were performed using Graph Pad Prism (GraphPad Inc, USA, version 5.02) software. Normality of the samples was assessed with the Kolmogorov-Smirnov test. One-way ANOVA followed by Newman-Keuls multiple comparison post-test were used for statistical comparisons of each pathologic line in a comparison test or Dunn’s multiple comparison post-test were used for statistical comparisons of each pathologic line against its respective driver control line and (or) transgenic control line. The smallest significance of both comparisons is reported on the graphs. The results are reported as mean ± standard error of the mean (SEM), with $P < 0.05$ considered as statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGMENTS

We thank M. Simonelig and N. Charlet for helpful comments as well as T. Cooper and A. Berglund for kindly providing plasmids.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a European FP6 grant No. 511978 to the network of excellence Myoress, by the ANR grant ‘MYO-ID’ (ANR-09-BLAN-0279), the Infrastructure grant TEFOR (ANR-11-INSB-0014), the FRM grant ‘Equipe FRM’ and AFM (15845) grants to K.J.

REFERENCES


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