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

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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 ion channel-1 (*ClC-I*) (11), Bin1 (12) and troponin T (*cTNT*) (13) are involved in insulin resistance, myotonia, muscle weakness and reduced myocardial

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function observed in patients. However, recent reports (14,15)
indicate that other molecular aberrations such as altered matu-
ration of miRNAs or CUG repeat-dependent transcription
factors leaching can also contribute to the pathogenesis of
DM1. To characterize molecular defects underlying this path-
ology, 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 pathogen-
esis (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 ex-
pression 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 demon-
strate 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 tran-
scriptomic 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 deregu-
lation is splice-independent, we found that the reduced transcript
levels of Muscle protein 20 (Mp20) gene were involved in myo-
blast fusion defects observed in DM1 larva. We also found
dSERCA as a target of Mbl and showed that the mis-
splicing of dSERCA transcripts leads to a decrease of its trans-
membrane 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) trans-
genic Drosophila lines carrying 240, 480, 600 and 960 inter-
rupted 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 gen-
erated 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 devel-
opmental 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 DM1 180 line, only few foci
were detected in DM1 480 (not shown) or DM1 600 lines,
whereas numerous foci were present in the DM1 960 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 musculature 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 in 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, re-
spectively, 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 dis-
played 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 muscle nuclei. No foci could be detected in Mef > 240CTG muscle nuclei, 1–2 foci could be detected in Mef > 600CTG muscle nuclei and several foci are visualized in Mef > 960CTG muscle nuclei. (C) Mef > 960CTG larval muscle nuclei after immunofluorescence and in situ hybridization. Mbl protein in nuclei stained with DAPI (blue) was detected by immunostaining (green) and co-localized with nuclear foci visualized with Cy3-labeled CAG probe (red). (D) RT–qPCR indicates that small RNAs expressed against mbl decreased by 50% global mbl transcript expression in Mef > mblRNAi third instar larvae. (E) Immunostaining against Mbl shows that Mbl was normally expressed in larval somatic muscle nuclei in the WT context, whereas Mbl expression could no longer be detected in larval somatic muscle nuclei in the Mef > mblRNAi line. (F) Average percentage of dead embryos and dead larvae are represented with SEM for each transgenic line (Mef > GFP, n_351 embryos = 351, n_L1_larvae = 172; Mef > mblRNAi, n_351 embryos = 142, n_L1_larvae = 105; Mef > 240CTG, n_351 embryos = 246, n_L1_larvae = 120; Mef > 600CTG, n_351 embryos = 528, n_L1_larvae = 236; Mef > 960CTG, n_351 embryos = 102, n_L1_larvae = 128). *P = 0.050 versus UAS-mblRNAi/+.

Figure 1. Validation of a new site-specific larval Drosophila model of DM1 and Mbl attenuated line. (A) RT–qPCR shows that site-specific third instar larvae expressing 240 CUG, 600 CUG and 960 CUG repeats in somatic muscles produce the same level of CUG transcripts. (B) In situ hybridization using a Cy3-labeled CAG probe was performed on larval muscles expressing GFP. Nuclei were stained with DAPI. CUG repeats accumulated in nuclear foci (red) in a size-dependent manner. No foci could be detected in Mef > 240CTG muscle nuclei, 1–2 foci could be detected in Mef > 600CTG muscle nuclei and several foci are visualized in Mef > 960CTG muscle nuclei. (C) Mef > 960CTG larval muscle nuclei after immunofluorescence and in situ hybridization. Mbl protein in nuclei stained with DAPI (blue) was detected by immunostaining (green) and co-localized with nuclear foci visualized with Cy3-labeled CAG probe (red). (D) RT–qPCR indicates that small RNAs expressed against mbl decreased by 50% global mbl transcript expression in Mef > mblRNAi third instar larvae. (E) Immunostaining against Mbl shows that Mbl was normally expressed in larval somatic muscle nuclei in the WT context, whereas Mbl expression could no longer be detected in larval somatic muscle nuclei in the Mef > mblRNAi line. (F) Average percentage of dead embryos and dead larvae are represented with SEM for each transgenic line (Mef > GFP, n_351 embryos = 351, n_L1_larvae = 172; Mef > mblRNAi, n_351 embryos = 142, n_L1_larvae = 105; Mef > 240CTG, n_351 embryos = 246, n_L1_larvae = 120; Mef > 600CTG, n_351 embryos = 528, n_L1_larvae = 236; Mef > 960CTG, n_351 embryos = 102, n_L1_larvae = 128). *P = 0.050 versus UAS-mblRNAi/+.
but rather result from the affected integrity of growing muscles, leading to their fragmentation during larval stages. In the case of the DM1960 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 DM1960 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 the affected integrity of growing muscles, leading to their fragmentation during larval stages.

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 larval 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 ~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 Cyp304a1 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 in microarray data apart from Cyp6a18 which is up-regulated. (E) Venn diagram showing transcript distribution between Mef \(-600\)CTG larvae (DM1\(_{600}\)) and Mef \(-960\)CTG larvae (DM1\(_{960}\)). 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 (DM1\(_{960}\) specific, DM1\(_{600}\) 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 (DM1\(_{960}\) specific \(n = 92\); DM1\(_{600}\) 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 DM1\(_{960}\) 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 \(-600\)CTG versus Mef \(-960\)CTG conditions (Mann–Whitney test).
often down-regulated (Fig. 5F). Among the GO classes that were over-represented when classifying DM1960 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 > 600CTG and Mef > 960CTG 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 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 DM1960 condition, we picked two high-ranked and evolutionarily conserved genes (Supplementary Material, Table S3b) from this category for validation: smvt (sodium-dependent multivitamin transporter) 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 DM1100 context (Fig. 5G). We then extended our analyses to two other highly ranked genes: Myosin Heavy Chain (Mhc) and upheld (up/ cTNT) (Supplementary Material, Table S3b) whose orthologs are already known to be affected in DM1 (13,31,32). Mhc and up code for major components of sarcomeres and as shown in Fig. 5G are down-regulated specifically in the DM1960 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: Muscle protein 20 (Mp20).

**Mp20** is known to be a positive modulator of myoblast fusion (33) during embryonic myogenesis. To validate decreased expression of **Mp20** detected by microarrays, we first performed a series of RT–qPCR experiments. We found that **Mp20** expression was indeed reduced in Mef > mblRNAi, Mef > 600CTG and in Mef > 960CTG 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 **Mp20** contributed to the affected myoblast fusion resulting in a reduced number of nuclei in larval muscles. We compensated for the reduced transcript levels of **Mp20** by targeting **Mp20** transgene expression to embryonic and larval muscles of mblRNAi and DM1 lines. The experiment performed showed that **Mp20** 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 *Drosophila* 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 *in silico* Mbl target genes producing transcripts that undergo alternative splicing was compared with splicing-dependent genes whose transcript level is altered in Mef > mblRNAi larvae. In all, 112 candidates were sorted out as common *in silico/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 mbl 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 *in silico/microarray* Mbl targets identified we found dSERCA encoding a calcium pump. Since *in silico* 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 isoform 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 DM1960 line and mbl attenuated line (Fig. 7E), but also in sarcomere for Mef > mblRNAi 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 mbl, 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 \textit{Mef}\textsubscript{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, \textit{Mef}\textsubscript{960CTG} and \textit{Mef}\textsubscript{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 \textit{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 \textit{Mef}\textsubscript{960CTG}, dSERCA\textsubscript{mb} and \textit{Mef}\textsubscript{mb}RNAi, dSERCA\textsubscript{mb} lines (Fig. 8A).

**DISCUSSION**

\textit{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 \textit{Mef} \textit{mblRNAi} line prevented comparative analyses with DM1 lines in adult flies.

\textbf{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 \textit{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 \textit{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 \textit{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 DM1\textsubscript{960} line. Surprisingly, the \textit{Mef} \textit{>240CTG} line which did not exhibit visible foci within muscle nuclei displayed altered motility associated with muscle splitting but
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 mbl attenuated lines display altered motility with affected complex movements. Interestingly, by measuring the contractility index and sarcomere size, we found that both the lines exhibited hypercontraction, a phenotype related to myotonia. It was previously shown that mbl1 disruption in the mouse also leads to myotonia (43). In our Drosophila DM1 model, we observed the effect of CTG repeat size on the severity of myotonia, so that the DM1600 line exhibits intermediate hypercontraction phenotypes when compared with DM1240 and DM1960. As not only hypercontraction but also affected myoblast fusion account for a reduced muscle size in pathological lines, we assume that both parameters need to be repaired to fully rescue muscle length.

**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).

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 DM1960 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.
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 (http://med.kuleuven.be/lcb/cisTargetX2%22) approach (48). 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). Interestingly, both qPCR and microarray experiments showed that Mp20

Table 1. Transcription factors potentially targeted by Mbl (in silico and i-cisTarget approaches)

<table>
<thead>
<tr>
<th>Transcription factors</th>
<th>Fold change</th>
<th>Total number of potential targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mef.mblRNAi</td>
<td>Mef.600CTG</td>
</tr>
<tr>
<td></td>
<td>versus MeFLacZ</td>
<td>versus MeFLacZ</td>
</tr>
<tr>
<td>pnr*</td>
<td>19.8006878</td>
<td>4.30132517</td>
</tr>
<tr>
<td>Srp</td>
<td>20.72330305</td>
<td>4.41837503</td>
</tr>
<tr>
<td>gm*</td>
<td>19.23811668</td>
<td>5.32013272</td>
</tr>
<tr>
<td>GATAAd</td>
<td>20.86058558</td>
<td>4.95156529</td>
</tr>
<tr>
<td>GATAe</td>
<td>21.60693721</td>
<td>2.29093816</td>
</tr>
<tr>
<td>Grh</td>
<td>2.526688665</td>
<td>1.91355347</td>
</tr>
<tr>
<td>GATAes</td>
<td>3.064233837</td>
<td>2.10105194</td>
</tr>
<tr>
<td>dMef2*</td>
<td>3.913588264</td>
<td>2.46400216</td>
</tr>
<tr>
<td>Ubx*</td>
<td>4.296620007</td>
<td>0.95861564</td>
</tr>
<tr>
<td>apa</td>
<td>0.285332972</td>
<td>1.04485069</td>
</tr>
<tr>
<td>Antp*</td>
<td>0.275286895</td>
<td>2.05870268</td>
</tr>
<tr>
<td></td>
<td>1.09052134</td>
<td>1.40489373</td>
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<td>1.566326296</td>
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<td></td>
<td>1.473393973</td>
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<tr>
<td></td>
<td>1.473393973</td>
<td>1.00018573</td>
</tr>
</tbody>
</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.
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 DM1960 larvae an of dSERCA in DM1-associated muscle contraction defect transcriptional profiling of in the transmembrane domain showed decreased expression in mbl attenuated and in DM1960 lines. This indicates that in the 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 forms devoid of the transmembrane domain for the DM1 larval muscles, in which the membrane-associated dSERCA 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 deletion 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-iCTG480 and UAS-iCTG600 plasmids were generated following published procedures (56). UAS-CTG660 was generated with repeats excised from a DMPK-CAG660 plasmid (13,57). All these constructs were generated by concatamerization 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-86Fh (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 transformation vector (26). The following pair of primers was used for amplification of Serca_mb sequence: 5’-atagcggccgctgaacgaa-3’ and 5’-atactcgagataatgaagttgggcttatgg-3’. The specific site P{Bac[y+]-attP-3B}VK00037 (BL#9752) host line was used for injection (Fly facility, Clermont-Ferrand, France).

w118: P{GD13374}v28732 line referred to as UAS-mbIRNAi (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{1} w{1}*; P[w+mC] = GAL4-Mef2.R3 mentioned as Mef-Gal4 line was ordered from the Bloomington Stock Center (BL#27390). The Mef > mCD8GFP line was a kind gift from A. Paulinat.
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′ 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 phalloidin-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 larvae 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: denaturating 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 ∆∆Ct 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 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.

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Conflict of Interest statement. None declared.

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