**LETTER**

**Targeting nuclear RNA for in vivo correction of myotonic dystrophy**

Thurman M. Wheeler1,2, Andrew J. Leger3, Sanjay K. Pandey4, A. Robert MacLeod4, Masayuki Nakamori1,2, Seng H. Cheng3, Bruce M. Wentworth3, C. Frank Bennett4 & Charles A. Thornton1,2

Antisense oligonucleotides (ASOs) hold promise for gene-specific knockdown in diseases that involve RNA or protein gain-of-function effects. In the hereditary degenerative disease myotonic dystrophy type 1 (DM1), transcripts from the mutant allele contain an expanded CUG repeat1–3 and are retained in the nucleus4,5. The mutant RNA exerts a toxic gain-of-function effect, making it an appropriate target for therapeutic ASOs. However, despite improvements in ASO chemistry and design, systemic use of ASOs is limited because uptake in many tissues, including skeletal and cardiac muscle, is not sufficient to silence target messenger RNAs7,8. Here we show that nuclear-retained transcripts containing expanded CUG (CUGexp) repeats are unusually sensitive to antisense silencing. In a transgenic mouse model of DM1, systemic administration of ASOs caused a rapid knockdown of CUGexp RNA in skeletal muscle, correcting the physiological, histopathologic and transcriptomic features of the disease. The effect was sustained for up to 1 year after treatment was discontinued. Systemically administered ASOs were also effective for muscle knockdown of Malat1, a long non-coding RNA (lncRNA) that is retained in the nucleus9. These results provide a general strategy to correct RNA gain-of-function effects and to modulate the expression of expanded repeats, lncRNAs and other transcripts with prolonged nuclear residence.

Antisense silencing by the RNase H-dependent mechanism entails a three-step process of oligonucleotide hybridization to its cognate RNA, cleavage of the target by RNase H1 and exonuclease degradation of the cleavage fragments. The relative efficiency of this mechanism in the nucleus and cytoplasm is uncertain. Observations that ASOs shuttle from cytoplasm to nucleus10 and that targeting intronic sequences to support RNase H activity (MOE gapmers; Supplementary Table 1) were sufficient to induce target cleavage.

To test this idea, we used a transgenic mouse model of DM1. HSA1LR transgenic mice express CUGexp RNA at high levels in skeletal muscle. Human DM1 is caused by an expanded CTG repeat in the 3' untranslated region (UTR) of dystrophia myotonica-protein kinase (DMPK)3, whereas in HSA1LR mice the expanded repeat is in the 3' UTR of a human skeletal actin (hACTA1) transgene6. In both conditions the CUGexp transcripts are retained in nuclear foci, along with splicing factors in the muscleblind-like (MBNL) protein family. Muscleblind sequestration leads to misregulated alternative splicing and other changes of the muscle transcriptome11–13. The RNA toxicity was mitigated in mice by CAG-repeat morpholino oligomers that competed with MBNL proteins for CUGexp binding, without activating RNase H. However, this approach required direct injection into a single muscle, followed by in vivo electroporation, a method to load muscle fibres with oligomers14. As an alternative, RNase H-active ASOs could produce widespread correction, provided that uptake of circulating ASOs was sufficient to induce target cleavage.

We identified ASOs showing a strong knockdown of hACTA1 in tissue culture, good tolerability when systemically administered in wild-type mice, and activity against hACTA1-CUGexp transcripts in vivo when electroporated into muscle (Supplementary Figs 1–3). The ASOs had 2'-O-methoxymethyl (MOE) modifications at both ends to maximize biostability, and a central gap of 10 unmodified nucleotides to support RNase H activity (MOE gapmers; Supplementary Table 1). We tested three of the ASOs in HSA1LR transgenic mice by subcutaneous injection of 25 mg kg−1 twice weekly (Fig. 1a). After 4 weeks of administration (8 injections), ASO 445236 reduced the level of CUGexp RNA in hindlimb muscles by more than 80% (Fig. 1b). Another ASO targeting the 3' UTR, downstream of the repeat tract, also showed strong CUGexp reduction, whereas an ASO targeting the 5' UTR, or three oligonucleotides against other targets, had no effect (Fig. 1b, c).

RNase H cleavage of mRNA is usually followed by rapid decay of cleavage fragments. However, stable cleavage fragments are observed occasionally15, and the CUGexp tract forms extensive hairpins16 and ribonucleoprotein complexes17 that could inhibit exonuclease activity. The failure of antisense targeting in the 5' UTR also raised the possibility that cleavage downstream of the repeat tract was required for efficient silencing. We therefore tested an additional ASO, 190401, targeting the hACTA1 coding region, and found that it also was highly effective (Fig. 1d). Furthermore, northern blot analysis using a CAG-repeat probe showed no evidence for a stable CUGexp cleavage fragment (Fig. 1e), and in situ hybridization showed reduction of nuclear CUGexp foci (Supplementary Fig. 4). These results indicate that expanded CUG repeats are degraded after a cleavage event 5' or 3' of the repeat tract.

Reduction of CUGexp RNA would be expected to release sequestered MBNL1 protein and improve its splicing regulatory activity. Consistent with this prediction, alternative splicing of four MBNL1-dependent exons, Sercal1 (also known as Atp2a1) exon 22, titin (Ttn) exon 362, Zasp (also known as Ldb3) exon 11, and Clcn1 chloride ion channel exon 7a, was normalized (Fig. 1f, g and Supplementary Figs 5 and 6)13. The Clcn1 splicing defect causes loss of channel function, repetitive action potentials and delayed muscle relaxation (myotonia)15,16, a cardinal feature of the disease. Blind analysis showed that myotonic discharges in hindlimb muscles were eliminated by the active ASOs (Fig. 1h), indicating rescue of Clcn1 function.

In addition to splicing defects, expression of CUGexp RNA or ablation of Mbnl1 causes extensive remodelling of the muscle transcriptome14,15,17,18. We used microarrays to examine transcriptomic effects of ASOs. Principle component analysis showed that gene expression in ASO-treated HSA1LR mice was shifted towards wild-type mice, indicating an overall trend for transcriptome normalization (Fig. 2a). Among transcripts that were up- or downregulated in HSA1LR muscle, more

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1Department of Neurology, University of Rochester, Rochester, New York, 14642, USA. 2Center for Neural Development and Disease, University of Rochester, 601 Elmwood Avenue, Rochester, New York, 14642, USA. 3Genzyme Corporation, 49 New York Avenue, Framingham, Massachusetts 01701, USA. 4Isis Pharmaceuticals, 2855 Gazelle Court, Carlsbad, California 92010, USA.

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Figure 1 | Systemic administration of 2′-O-(2-methoxyethyl) ASOs in the HSA\textsuperscript{LR} transgenic mouse model of DM1. a, Location of ASO-targeting sequences relative to the hACTA1 coding region and the expanded CUG repeat in the 3′ UTR. b, Quantitative real-time RT–PCR of hACTA1-CUG\textsuperscript{exp} mRNA in quadriceps (Quad), gastrocnemius (Gastroc) and tibialis anterior (TA) muscle in HSA\textsuperscript{LR} mice treated with the indicated ASOs by subcutaneous injection of 25 mg kg\textsuperscript{−1} twice weekly for 4 weeks. Muscle tissue was obtained 1 week after the final dose (4 per group). The mean levels of transgene mRNA ± s.d. are shown. ***P < 0.001, ****P < 0.0001 (one-way analysis of variance (ANOVA)). c, hACTA1-CUG\textsuperscript{exp} transcript levels in quadriceps are not affected by ASOs targeting unrelated transcripts (141923, randomer; 116847, Pten; 399462, Malat1); n = 4 per group; same dose as in b. Error bars are mean ± s.d. d, Knockdown of hACTA1-CUG\textsuperscript{exp} mRNA in muscle by ASO 190401 (n = 4 per group; same dose as in b). Error bars are mean ± s.d. ***P < 0.001 for ASO-treated versus saline-treated muscles (two-way ANOVA).

10 different CUG\textsuperscript{exp} mRNA targets. (Supplementary Fig. 9a–c), the latter muscle having higher basal levels of CUG\textsuperscript{exp} expression\textsuperscript{18}. Serum chemistries showed no evidence for renal or liver toxicity (Supplementary Fig. 10).

A uniform finding in previous studies of MOE gapmer ASOs was that systemic administration failed to cause significant target reduction in muscle, despite efficient knockdown in liver (n = 12 different mRNA targets; Supplementary Table 3), raising the possibility that muscle tissue in our model is unusually susceptible to antisense silencing. We examined the functional integrity of the muscle
membrane, a physiological barrier to ASO uptake\textsuperscript{44}, and found that muscle penetration of the extravascular dye, Evans Blue, was similar in wild-type and HSA\textsuperscript{LR} mice (Supplementary Fig. 11b). Direct analysis of muscle tissue indicated that ASO accumulation was no greater in HSA\textsuperscript{LR} mice than in wild-type controls (Supplementary Fig. 11b, c). Likewise, the mRNA level for RNase H1 was similar in HSA\textsuperscript{LR} and wild-type muscle (Supplementary Fig. 12). We tested ASOs targeting other muscle-expressed transcripts. ASOs for Pten phosphatase or Srb1 (also known as Scarb1) scavenger receptor showed efficient target knockdown in liver, but no appreciable knockdown in HSA\textsuperscript{LR} or wild-type muscle (Fig. 3a). Taken together with previous studies, our results indicate specific sensitivity of hACTA1-CUG\textsuperscript{exp} transcripts rather than a general enhancement of ASO activity in HSA\textsuperscript{LR} muscle.

A notable metabolic feature of hACTA1-CUG\textsuperscript{exp} and human DMPK-CUG\textsuperscript{exp} mRNA is that processing and polyadenylation are normal but the transcripts are retained in the nucleus\textsuperscript{6,8}. Recent studies have shown that RNase H1, the enzyme responsible for antisense knockdown, is localized to the nucleus and mitochondria\textsuperscript{44}, suggesting that antisense cleavage of nuclear-encoded RNA occurs before nuclear export, and raising the possibility that nuclear-retained transcripts may exhibit enhanced sensitivity. To determine whether other nuclear-retained transcripts show a similar effect we examined metastasis-associated lung adenocarcinoma transcript 1 (Malat1), an endogenous nuclear lncRNA\textsuperscript{35}. We identified MOE gapmer ASOs that produced strong Malat1 knockdown in cells, in an RNase H1-dependent manner (Supplementary Fig. 13). In wild-type and HSA\textsuperscript{LR} mice, subcutaneous administration of ASOs for 4 weeks caused a greater than 80% reduction in Malat1 in muscle (Fig. 3b, c), supporting the idea that nuclear-retained transcripts have enhanced sensitivity.

To determine the duration of ASO action in muscle, we examined mice at 15 and 31 weeks after ASO was discontinued, and found that hACTA1-CUG\textsuperscript{exp} knockdown and splicing correction remained strong (not shown). One year after ASO injection was discontinued, target reduction by ASO 190401 had waned, but remained approximately 50% or more for ASO 445236 (Fig. 4a). Even at this late time point the appropriate cleavage products were detected by amplification of complementary DNA 5’ ends (5’ RACE), indicating persistent ASO-RNase H1 activity (Fig. 4b). Consistent with the extent of target reduction, there was partial return of myotonia and splicing defects for

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**Figure 2** | Effects of ASOs on the transcriptome in quadriceps muscle. a. Principle component analysis of microarray data shows segregation of HSA\textsuperscript{LR} (saline) away from wild-type mice in widely separated clusters. ASOs caused HSA\textsuperscript{LR} transgenic mice to cluster nearer to wild-type mice (25 mg kg\textsuperscript{-1} biweekly for 4 weeks; n = 4 mice per group). b. Of the transcripts upregulated in HSA\textsuperscript{LR} versus wild-type mice (saline), >85% showed complete or partial return to normal expression after treatment with ASOs (n = 4 mice per group).

**Figure 3** | Differential sensitivity of transcripts to ASO knockdown in skeletal muscle. a. In HSA\textsuperscript{LR} or FVB/N wild-type mice, ASOs targeting Srb1 (353382) or Pten (116847) were effective for knockdown in liver but not in quadriceps muscle (qRT–PCR, n = 4 per group). Error bars are mean ± s.d. *P = 0.02, **P < 0.001 (t-test). b. HSA\textsuperscript{LR} and FVB/N wild-type mice were treated with ASO 399462 targeting Malat1, a nuclear-retained IncRNA. Levels of Malat1 transcript in the indicated tissues were determined by qRT–PCR (n = 4 ASO, 3 saline). Error bars are mean ± s.d. *P = 0.035, **P < 0.007, ***P = 0.001 for ASO versus saline (t-test). c. Dose response of Malat1 knockdown in BALB/c wild-type mice. BALB/c wild-type mice were treated with saline or ASO 399462 targeting Malat1 at 12.5, 25 and 50 mg kg\textsuperscript{-1} twice per week for 3.5 weeks (7 doses in total; n = 4 per group). Tissues were collected for RNA isolation 2 days after the final dose. Malat1 transcript levels were determined by qRT–PCR. Error bars are mean ± s.e.m. *P < 0.01, **P < 0.001, ***P < 0.0001 (two-way ANOVA).
ASO 190401, whereas correction by ASO 445236 remained strong (Fig. 4e, d and Supplementary Fig. 14a–e). Furthermore, the persistent knockdown of CUG<sup>sp</sup> RNA largely prevented the age-dependent myopathic changes in HSALR muscle, as evidenced by reduced frequency of central nuclei (Fig. 4e) and improved muscle-fibre diameter (mainly a prevention of fibre atrophy) (Supplementary Fig. 15). These findings indicate that ASO activity against hACTA1-CUG<sup>sp</sup> in muscle is remarkably durable and that long-term reduction of the toxic RNA can protect against structural changes in muscle fibres. Notably, the duration of Malat1 knockdown in muscle was also prolonged (a greater than 50% reduction at 31 weeks after ASO discontinuation) and more persistent than in liver (Supplementary Fig. 16).

Therapeutic application of this strategy to human DM1 will require transfer of the targeting sequence to hDMPK. We developed MOE gapmer ASOs that were active against hDMPK in cells. We examined in vivo activity after 4 weeks of twice weekly subcutaneous injection in transgenic mice that express hDMPK with 800 CUG repeats. The ASO produced significant knockdown of hDMPK-CUG<sup>exp</sup> transcripts in hindlimb muscle (Fig. 4f and Supplementary Figs 17 and 18), supporting the feasibility of silencing the pathogenic DMPK allele.

Despite physiological barriers to tissue uptake, our results indicate that systemic targeting of CUG<sup>sp</sup> RNA is feasible because small amounts of ASOs that enter muscle fibres can hybridize their target and productively engage RNase H1. Although the mechanisms for enhanced sensitivity of CUG<sup>sp</sup> RNA and Malat1 are not fully defined, our data suggest that residence time in the nucleus is an important determinant of transcript sensitivity. Features of the nuclear environment that may enhance antisense activity include nuclear localization of RNase H1 (ref. 14) and auxiliary proteins that promote oligonucleotide hybridization<sup>25</sup>, and—in the case of CUG<sup>exp</sup> transcripts—spatial concentration of targets in a small volume. A similar approach may be effective for other genetic disorders that have nuclear accumulation of repeat expansion RNA<sup>26–27</sup>. Previous studies have used CAG-repeat ASOs that bind CUG<sup>sp</sup> RNA without activating RNase H, in an effort to block the protein interactions or modify the metabolism of the toxic RNA<sup>18,28</sup>. Although this approach was effective with local delivery, initial attempts at systemic delivery were less successful (T.M.W. and C.A.T., unpublished observations), which fits with the expectation that higher tissue concentrations of ASO are required to occupy CUG<sup>exp</sup> binding sites than to induce RNase H cleavage. Furthermore, the RNase H mechanism is attractive because it exploits the nuclear retention phenomenon to gain a therapeutic advantage, while posing less risk of off-target effects by avoiding a repetitive sequence. Recently, local delivery of RNase H-active CAG-repeat ASOs induced partial CUG<sup>sp</sup> knockdown, but was accompanied by muscle damage<sup>29</sup>, again suggesting that direct targeting of the repeat tract may have pitfalls. Our results also suggest that ASOs are useful for in vivo functional characterization and therapeutic modulation of lncRNAs, a large and recently recognized class of regulatory RNAs<sup>30</sup>.

**METHODS SUMMARY**

**Experimental mice.** All animal experiments were approved by the Institutional Animal Care and Use Committees at the University of Rochester, Genzyme Corporation and Isis Pharmaceuticals.

**Subcutaneous injection of ASOs.** MOE gapmer ASOs were dissolved in saline and administered by subcutaneous injection in the interscapular region twice per week at the indicated doses.

**Quantitative real-time RT–PCR (polymerase chain reaction with reverse transcription) assay.** Total RNA was purified from muscle using RNeasy Lipid Tissue Mini Kits (Qiagen). mRNA levels for ACTA1, Srb1, Pten, Malat1 and RNase H1 were determined on the Applied Biosystems 7500 System using 18S rRNA as normalization control. General transcription factor 2b (Gtf2b) and total RNA (Ribogreen assay) served as normalization controls for human DMPK and mouse Dmpk.

**Northern analysis.** CUG<sup>sp</sup> sequences were detected using a 32P end-labelled (CAG)<sub>d</sub> DNA oligonucleotide probe.

**Electromyography.** Electromyography (EMG) was carried out blind under general anaesthesia, as described previously<sup>14</sup>. RT–PCR analysis of alternative splicing. RT–PCR was carried out using the SuperScript III One-Step RT–PCR System with Platinum Taq DNA Polymerase (Invitrogen) and the same gene-specific primers for cDNA synthesis and PCR amplification. PCR products were separated on agarose gels, stained with SyBrGreen I Nucleic Acid Gel Stain (Invitrogen) and scanned with a fluorimeter. **Transcriptome analysis.** Quadriceps-muscle RNA from wild-type or HSALR transgenic mice treated with vehicle (saline), ASO 445236 or ASO 190401 was processed to cRNA and hybridized on microarrays using MouseRef-8 v2.0 Expression BeadChip Kits (Illumina). Image data were quantified using BeadStudio software (Illumina).
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Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions T.M.W., A.J.L., S.K.P., A.R.M., M.N., S.H.C., B.M.W., C.F.B. and C.A.T. participated in the planning, design and interpretation of experiments. T.M.W., A.J.L., S.K.P., A.R.M and M.N. carried out experiments. T.M.W. and C.A.T. wrote the manuscript.

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METHODS
Antisense oligonucleotides. ASOs were synthesized at Isis Pharmaceuticals, as described previously. All ASOs were MOE ganoon 20mers with phosphorothioate as the internuslink unit, 2'-O-(2-methoxyethyl) (MOE) modifications of 5 nucleotides at the 5' and 3' end, and a central gap of 10 deoxynucleotides. The sequence of each ASO is listed in Supplementary Table 1. CAG25 and GAC25 morpholinos were purchased (Gene Tools).

Identification of active ASOs. The criteria for identifying active hACTA1-targeting ASOs were as follows: first, selection of targeting sequences that were not conserved in mice, to avoid knockdown of endogenous skeletal actin; second, >30% hACTA1 knockdown when electroporated in HepG2 cells (Supplementary Fig. 1); and third, absence of hepatotoxic or immunostimulatory effects in wild-type mice, when 50 mg kg⁻¹ was injected subcutaneously twice weekly for 4 weeks (Supplementary Fig. 2a–c). Out of 11 candidate ASOs examined, 5 satisfied these criteria. For the ASO with the highest activity in HepG2 cells, we also verified activity against hACTA1-CUG₉₉ transcripts in vivo, by direct injection and electroporation of tailbasius anterior muscle in HSA₁₈ mice (Supplementary Fig. 3). Four of the five ASOs were subsequently used for subcutaneous administration in HSA₁₈ mice. ASOs targeting Malat1 were identified by demonstration of >50% target knockdown when electroporated in mouse hepatocellular SV40 large T-antigen carcinoma (MHT) cells, and absence of hepatotoxic or immunostimulatory effects in wild-type mice (dosing as above).

Cell transfection and gene analysis. HepG2 cells were electroporated in a 96-well plate format at 165V with ASOs in complete media containing MEM, non-essential amino acid (NEAA), sodium pyruvate and 10% FBS at room temperature. Cells were incubated overnight and lysed in RLT buffer (Qiagen). Total RNA was purified using Qiagen RNeasy kit. Quantitative real-time RT–PCR (qRT–PCR) was performed using the Qiagen Quantitect Probe RT–PCR kit. Twenty-microtitre qRT–PCR reactions were run in duplicate and normalized against against human RNA, calculated using the Ribogreen assay (Invitrogen).

Experimental mice. Institutional Animal Care and Use Committees at the University of Rochester, Genzyme Corporation and Isis Pharmaceuticals approved all animal experiments. HSA₁₈ mice in the line 20b were derived and backcrossed to C57Bl/10 mice. Twenty-microlitre qRT–PCR reactions were run in duplicate and normalized with 18S rRNA as normalization control, according to the manufacturer's recommendations. Image data were quantified using BeadStudio software (Illumina). Signal intensities were quantile normalized. We used row-specific off-sets to avoid any values of less than two, before the normalization. Data from all probe sets with six or more nucleotides of CUG, UGC or GCU repeats were suppressed to eliminate the possibility that expanded repeats in the hybridization mixture (CAG repeats in cRNA, originating from CUG₉₉ RNA) could cross-hybridize with repeat sequences on probes. To eliminate genes whose expression was not readily quantified on the arrays, we suppressed probes that did not show a detection probability of P ≤ 0.1 for all samples in the group that showed the higher mean expression level. Comparisons between groups were summarized and ranked by fold-changes of mean expression level and t-tests. The software package R (ref. 38) was used to perform principal components analysis (PCA) on wild-type, ASO-treated, and saline-treated microarray samples. The principal components allowed the capture of the majority of the expression variation in each sample within three dimensions. We plotted the first three principal components of each sample. Array data have been submitted to the Gene Expression Omnibus, accession number GSE38962 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38962).

Fluorescence in situ hybridization. Localization of CUG₉₉ RNA by fluorescence in situ hybridization (FISH) was carried out using a CAG repeat oligoribonucleotide probe labelled with Texas Red at the 5' end, on muscle cryosections from ASO- or saline-treated mice, as described previously. Images are maximum projections of deconvolved Z-plane stacks (9 images, 0.1- or 0.2-μm steps) captured under identical exposure and illumination conditions using a fluorescence microscope (Carl Zeiss Axioplan 2 or Nikon Eclipse E600), a charge-coupled device (CCD) digital camera (Hamamatsu ORCA R2 or Photometrics Cool Snap HQ) and Metamorph software (Molecular Devices). The Optigrid structured illumination imaging system (Quioptiq) was also used to capture images of DM328XL muscle. Maximum grey-level intensity was quantified using Metamorph. Objectives of ×100 Plan-APochromat 1.4 NA oil (Zeiss) or ×60 Plan Apo 1.4 NA oil (Nikon).

Muscle fibre morphology. To outline muscle fibres and label nuclei, 10-μm transverse cryosections of muscles from ASO- or saline-treated mice were fixed with 4% paraformaldehyde, pH 7.3, washed in PBS and incubated in 20 μg ml⁻¹ dihydroxybenzidine (DAB) and 5% hydrogen peroxide in PBS. After washing in PBS, muscles were incubated in 20 μg ml⁻¹ DAPI for 1 h at room temperature. Sections then were washed in PBS, mounted and sealed. Images were captured using an Axioplan 2 fluorescence microscope (Zeiss), an ORCA R2 CCD digital camera (Hamamatsu Photonics), Metamorph software and a ×20 Plan-Neofluar 0.5 NA objective (Zeiss). Using the calipers application in Metamorph, the muscle fibre diameter, defined as the minimum 'Feret's diameter' (the minimum distance of parallel tangents at opposing borders of the muscle fibre), was determined. Haematoxylin and eosin (H&E)-stained images were captured using an Infinity 2 1.4 megapixel colour CCD digital camera (Lumenera), Infinity Analyze 5.0 software (Lumenera) and Axiovision software (Zeiss) as described previously (7). 5' rapid amplification of cDNA ends analysis. 5' rapid amplification of cDNA ends (RACE) was carried out using the FirstChoice RLM-RACE Kit (Invitrogen). In brief, 1 μg of total mRNA was ligated with 5' RACE adaptor (5′-GGCUAGUUGGGAUGAAGAACACUCCUGUUUGCUUGCCAGUAGA)
AA-3’), then reverse transcribed with a primer specific for the cleavage fragment (5’-TGGAGAAGTGCCGTCGGA-3’ for 190401, or 5’-TTTTTTTTTACGCA GC-3’ for 445236). The synthesized cDNA was treated with RNase H, then amplified with 5’ RACE Outer Primer and 5’-TTGGGTGAGCATGGAAGG-3’ (for 190401 fragment), or 5’-TGTGTAAACGACGGCAATGCAGCAGGTTA ACAGAATGAC-3’ (for 445236 fragment). The PCR products were analysed on agarose gels stained with SYBR Green I (Invitrogen) and scanned with a laser fluorimeter (Typhoon, GE Healthcare).

RNAse H1 short interfering RNA experiments. MHT cells were cultured in DMEM supplemented with 10% fetal calf serum, streptomycin (0.1 mg ml⁻¹), and penicillin (100 U ml⁻¹). Short interfering RNA (siRNA) treatments were carried out using Opti-MEM containing 5 mg ml⁻¹ Lipofectamine 2000, as previously described. In brief, MHT cells were plated with 7,500 cells per well and were incubated for either 24 or 48 h with 75 nM of siRNA targeting RNAseH1 (5’-GGCTTTGGTAGACGTGTTATTT-3’ and 5’-TAACGACGTCTCAGCCAAGCTG-3’) or Ap2M1 (sequences reported previously in OPTI-MEM and Lipofectamine 2000. Twenty-four hours post transfection, cells were treated with increasing doses of the Malat1-targeting ASO 399479 in DMEM–10% FBS. Twenty-four hours after the addition of oligonucleotides, cells were lysed and RNA was isolated using RNeasy and qRT–PCR was performed to determine the level of Malat1 mRNA.

Tissue drug-level determination. Approximately 30 to 100 mg liver and muscle tissue were homogenized as described. Capillary gel electrophoresis (CGE) methods were used to measure unlabelled drug concentrations in mouse tissues. The methods for the hACTA1 ASOs were slight modifications of previously published methods. A normal concentration of 10 mg ml⁻¹ Lipofectamine 2000 was used for the internal standard (ASO 190401 fragment, or 445236 fragment). The PCR products were added before extraction. Tissue sample analyses were conducted using a Beckman MDQ capillary electrophoresis instrument (Beckman Coulter). Biochemical analysis and serum chemistry. Serum separated in serum separator tubes (BD catalogue number 365956) was used to determine aspartate transaminase and lactate dehydrogenase activity. Serum was analysed using Opti-MEM containing 5 mg ml⁻¹ Lipofectamine 2000, as previously described. In brief, MHT cells were plated with 7,500 cells per well and were incubated for 24 or 48 h with 75 nM of siRNA targeting RNAseH1 (5’-GGCTTTGGTAGACGTGTTATTT-3’ and 5’-TAACGACGTCTCAGCCAAGCTG-3’) or Ap2M1 (sequences reported previously in OPTI-MEM and Lipofectamine 2000. Twenty-four hours post transfection, cells were treated with increasing doses of the Malat1-targeting ASO 399479 in DMEM–10% FBS. Town four hours after the addition of oligonucleotides, cells were lysed and RNA was isolated using RNeasy and qRT–PCR was performed to determine the level of Malat1 mRNA.

Biochemical analysis and serum chemistry. Serum separated in serum separator tubes (BD catalogue number 365956) was used to determine aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine values using Olympus reagents and an Olympus AU400e analyser (Melville). Evans blue dye uptake assay. Evans blue dye (EBD) was dissolved in PBS at a concentration of 10 mg ml⁻¹ and filter-sterilized. HSA, FVB/N, Mdx or C57Bl/10 mice were administered an intraperitoneal injection of 10 μl EBD solution per gram of bodyweight. After a period of 24 h, muscle tissues were collected (quadriceps, gastrocnemius, tibialis anterior, diaphragm and heart). The mass of each muscle was recorded before lysing each sample individually in a microfuge tube containing N,N-dimethylformamide and a 5-mm steel bead, which was subjected to 30 Hz shaking in a Qiagen TissueLyser II. Lysed muscle samples were heated at 55 °C and centrifuged, and the absorbance of the supernatant was determined by spectrophotometric measurement at 636 nm. A standard curve of EBD in N,N-dimethylformamide enabled the EBD content in individual muscle samples to be determined.

Statistical analysis. Group data are presented as mean ± s.d., except where mean ± s.e.m. is indicated. Between-group comparison was carried out using a two-tailed Student’s t-test or an analysis of variance (ANOVA), as indicated. A P value of <0.05 was considered significant.