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Correspondence and requests for materials should be addressed to S.I. (cishiura@mail.ecc.u-tokyo.ac.jp)

Manumycin A corrects aberrant splicing of *Clcn1* in myotonic dystrophy type 1 (DM1) mice

Kosuke Oana[1], Yoko Oma[1], Satoshi Suo[1], Masanori P. Takahashi[2], Ichizo Nishino[3], Shin'ichi Takeda[4] & Shoichi Ishiura[1]

[1]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Tokyo, Japan, [2]Department of Neurology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, [3]Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan, [4]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan.

Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults and as yet no cure for DM1. Here, we report the potential of manumycin A for a novel DM1 therapeutic reagent. DM1 is caused by expansion of CTG repeat. Mutant transcripts containing expanded CUG repeats lead to aberrant regulation of alternative splicing. Myotonia (delayed muscle relaxation) is the most commonly observed symptom in DM1 patients and is caused by aberrant splicing of the skeletal muscle chloride channel (*CLCN1*) gene. Identification of small-molecule compounds that correct aberrant splicing in DM1 is attracting much attention as a way of improving understanding of the mechanism of DM1 pathology and improving treatment of DM1 patients. In this study, we generated a reporter screening system and searched for small-molecule compounds. We found that manumycin A corrects aberrant splicing of *Clcn1* in cell and mouse models of DM1.

Myotonic dystrophy (DM), a genetic disorder, is the most common type of muscular dystrophy in adults[1]. The types of DM disease[2], DM types 1 (DM1) and 2 (DM2), differ genetically but are similar clinically. DM1 is caused by expansion of a CTG repeat in the 3′-untranslated region (UTR) of the DM protein kinase (*DMPK*) gene, whereas DM2 is caused by expansion of a CCTG repeat in intron 1 of the zinc finger 9 (*ZNF9*) gene[3–5]. DM1 presents with a variety of symptoms, such as myotonia, progressive muscle wasting, cataracts, insulin resistance, and intellectual deficits[1,6]. Identification of the DM2 mutation[5] and studies using DM1 mice that express the expanded CUG repeat[7] suggest that RNA gain-of-function causes the DM1 phenotype.

How does nucleotide expansion within a non-coding region cause DM1? An investigation of the molecular mechanism of DM1 proposed that expanded repeat RNA transcripts form hairpin structures that retain nuclear foci in DM1 cells[8], and affect the function of RNA-binding proteins[9–12]. 'Muscleblind-like' (MBNL) and 'CUGBP and ETR-3 like factor' (CELF) proteins are well-studied RNA-binding proteins. Sequestration of MBNL1 by toxic expanded RNA transcripts[11,13] and up-regulation of CUGBP-1[14] result in aberrant regulation of alternative splicing events in DM1[2]. Our previous studies indicated aberrant regulation of *MYOM1* and *PDLIM3* in DM1 patients[15,16]; more than 25 genes have been found to be misregulated in patients with DM[17]. Missplicing of the following genes has been associated with DM1 symptoms: *CLCN1*, which results in myotonia[7,18], *BIN1*, which is associated with T-tubule alterations and muscle weakness[19], and *INSR*, which contributes to insulin resistance[20]. However, there is no evident relationship between additional misspliced genes and DM1 symptoms. Therefore, further studies are needed to elucidate this association.

Myotonia is one of the features observed most commonly in individuals with DM1. People with myotonia are unable to relax certain muscles after use. For example, a person may not be able to release their grip on a doorknob or handle. In DM1 patients, the inclusion of alternative exons 6B and/or 7A, and retention of intron 2 of *CLCN1*, are observed due to aberrant regulation of alternative splicing[18]. Abnormal splicing of *CLCN1* results in a frameshift and produces premature termination codons in transcripts, leading to nonsense-mediated mRNA decay (NMD) or the production of a truncated protein with a dominant-negative effect[21]. A mouse model of DM1 expressing an expanded CUG repeat (HSA^{LR}) also shows increased inclusion of *Clcn1* exon 7A and displays

myotonia[7]. Our previous study using a *Clcn1* minigene identified regulation of exon 7A by MBNL and CELF proteins[22].

The identification of small-molecule compounds that correct missplicing events in DM1 would benefit both our understanding of novel aspects of DM1 pathogenesis and DM1 therapy. In DM1, there may be other key players in addition to MBNL and CELF. There are several approaches to DM1 therapy, such as overexpression of MBNL1[23], RNA interference targeting CUG repeat transcripts[24], inhibition of MBNL1 sequestration through use of a CAG oligonucleotide that binds to the CUG repeats[25] or a small molecule[26], and degradation of expanded CUG repeat transcripts through the RNase H pathway, which occurs through induction of 2′ methoxyethyl (MOE) gapmers[27]. Although use of an antisense oligonucleotide showed remarkable effects, there was a difficulty with body-wide delivery while small-molecule compounds have the advantage of oral formulation.

In this study, we established a Clcn1-L minigene reporter assay and found that manumycin A corrects abnormal splicing of *Clcn1*. Furthermore, we confirmed that injection of manumycin A corrects missplicing of *Clcn1* in a mouse model of DM1 via H-Ras pathway.

Results

Generation of the Clcn1-L reporter assay system. To identify small chemical compounds effective against aberrant splicing of *CLCN1* in DM1, we generated a minigene reporter vector containing the mouse *Clcn1* gene from exons 6 to 7 and the firefly luciferase gene (Fig. 1a). As shown in Fig. 1b, luciferase expression was obtained upon exclusion of exon 7A; however, inclusion of exon 7A produced a termination codon, resulting in a lack of luciferase expression. Next, we confirmed the co-transfection of Clcn1-L and DMPK constructs harboring either CTG18 (DM18) or interrupted CTG480 (DM480) repeats. RT-PCR analysis revealed that inclusion of exon 7A was significantly increased upon co-expression of Clcn1-L and DM480 compared to co-expression with DM18 (Fig. 1c,d). According to the luciferase analysis, co-expression of Clcn1-L and DM18 displayed a higher activity than did co-expression with DM480 (Fig. 1e). Note that we did not delete the initiation codon of luciferase gene. It is possible that an extra ATG codon in the Clcn1-L might induce translation of luciferase irrespective of exon 7A exclusion. However, in our experiments, the luciferase activity decreased when DM480 was transfected (Fig. 1e). Therefore, we concluded that this reporter system was properly working.

Identification of small-molecule compounds that correct aberrant splicing of Clcn1 under the expression of expanded CUG repeats in vitro. The ICCB Known Bioactives Library is a collection of compounds with defined biological activities. The library was screened to identify compounds that corrected aberrant splicing of *Clcn1* using our luciferase reporter assay. Because expanded CUG repeat expression causes aberrant regulation of alternative splicing in DM1, we transfected C2C12 cells with both the Clcn1-L luciferase reporter vector and DM480, which expresses the expanded CUG repeat. Although most compounds showed little effect compared to that of DMSO treatment (control, Table S1), some of the compounds showed high luciferase activity (Fig. S1), and Ro 31-8220, AGC, and manumycin A caused little toxicity to cells (Fig. S2). Since Ro 31-8220 was well studied with DM1[28] and AGC caused large SEM, manumycin A was chosen in this study for further analysis. Manumycin A (20 μM) showed high luciferase activity in the presence of the expanded CUG repeat (Fig. 2a and Fig. S1).

Next, we performed RT-PCR analysis to investigate whether manumycin A corrects aberrant *Clcn1* splicing caused by expression of the expanded CUG repeats. Results from RT-PCR showed that the addition of manumycin A effectively corrects aberrant splicing of *Clcn1* in the presence of the expanded CUG repeat (Fig. 2b,c). Percentages of *Clcn1* exon 7A inclusion showed that manumycin A treatment rescued abnormal exon 7A inclusion levels caused by expression of the expanded CUG repeat to levels similar to those for the normal CUG repeat (Fig. 2c). Additionally, we tested the dosage of manumycin A (~10-40 μM), and found that the effects of manumycin A were concentration-dependent (Fig. S3). High-dosage manumycin A rescued aberrant splicing in the presence of the expanded CUG repeat and showed skipping of exon 7A (Fig. S3) compared to the control (Fig. 2c).

Manumycin A corrects aberrant splicing of Clcn1 in a mouse model of DM1. Next, we examined the ability of manumycin A to rescue aberrant splicing of *Clcn1* in a mouse model (HSA^{LR}) of DM1, in which 250 CUG repeats are expressed under the control of the actin promoter. RT-PCR analysis showed elevated inclusion of *Clcn1* exon 7A in the HSA^{LR} mice compared with the wild-type mice (Fig. 3a,b). Injection of manumycin A induced a remarkable reduction in *Clcn1* exon 7A inclusion (Fig. 3c,d). However, manumycin A did not rescue aberrant splicing of *Serca1* and m-Titin (Fig. S4).

H-Ras regulates alternative splicing of Clcn1 exon 7A. Manumycin A is an antibiotic generated by *Streptomyces parvulus*[29]. It acts as a selective and vigorous inhibitor of Ras farnesyltransferase[30]. After translation, Ras protein requires several modifications: isoprenylation, proteolysis, methylation and palmitoylation[31–33]. Isoprenylation by the enzyme farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) is the first step in the post-translational modification of Ras. Farnesylation or geranylgeranylation is necessary for Ras to attach to the inner side of the plasma membrane. Without attachment to the cell membrane, Ras is unable to be activated[34]. H-Ras, K-Ras and N-Ras are the members of the Ras family. It is important that H-Ras is only farnesylated, whereas K-Ras and N-Ras can be farnesylated and geranylgeranylated. Thus, inhibitors of farnesyltransferase are effective in reducing the activity of H-Ras but not that of K-Ras and N-Ras[35]. Correction of *Clcn1* splicing by manumycin A may therefore be due to the inhibition of H-Ras activity. To test this possibility, we knocked down endogenous H-Ras expression using small interfering RNA (siRNA) and examined the effect of H-Ras inhibition on *Clcn1* splicing. We confirmed the efficacy of the siRNA in modulating the expression of the H-Ras by Western blot analysis (Fig. 4a). RT-PCR analysis showed reduced inclusion of *Clcn1* exon 7A in the presence of expanded CUG repeats (Fig. 4b,c). We also examined the effects of K-Ras and N-Ras knockdown and found that N-Ras knockdown reduced the inclusion of *Clcn1* exon 7A, whereas K-Ras knockdown did not alter *Clcn1* splicing (Fig. S5). Additionally, we tested whether expanded CUG repeats altered the expression levels of H-Ras. Although there was no significant difference between DM18- and DM480-transfected C2C12 cells, an upward trend was observed when cells were transfected with DM480 (Fig. S6).

Discussion

Misregulation of alternative splicing is a characteristic feature of DM1. Although the number of missplicing events is over 25, few genes have been reported to play a role in disease manifestations. Abnormal regulation of alternative splicing in the *CLCN1* gene is one of the events that can account for myotonia, which is often recognized in DM1. In this investigation, we generated a Clcn1-L reporter assay system and searched for small-molecule compounds that affect abnormal splicing of *Clcn1* in the presence of expanded CUG repeats. We found that manumycin A corrects aberrant splicing of *Clcn1*, which was confirmed *in vivo*. We also revealed that H-Ras was involved in the regulation of alternative splicing of *Clcn1* exon 7A.

Injection of DM1 model mice with manumucin A corrected aberrant splicing of *Clcn1*; however, splicing of *Serca1* and m-Titin, which

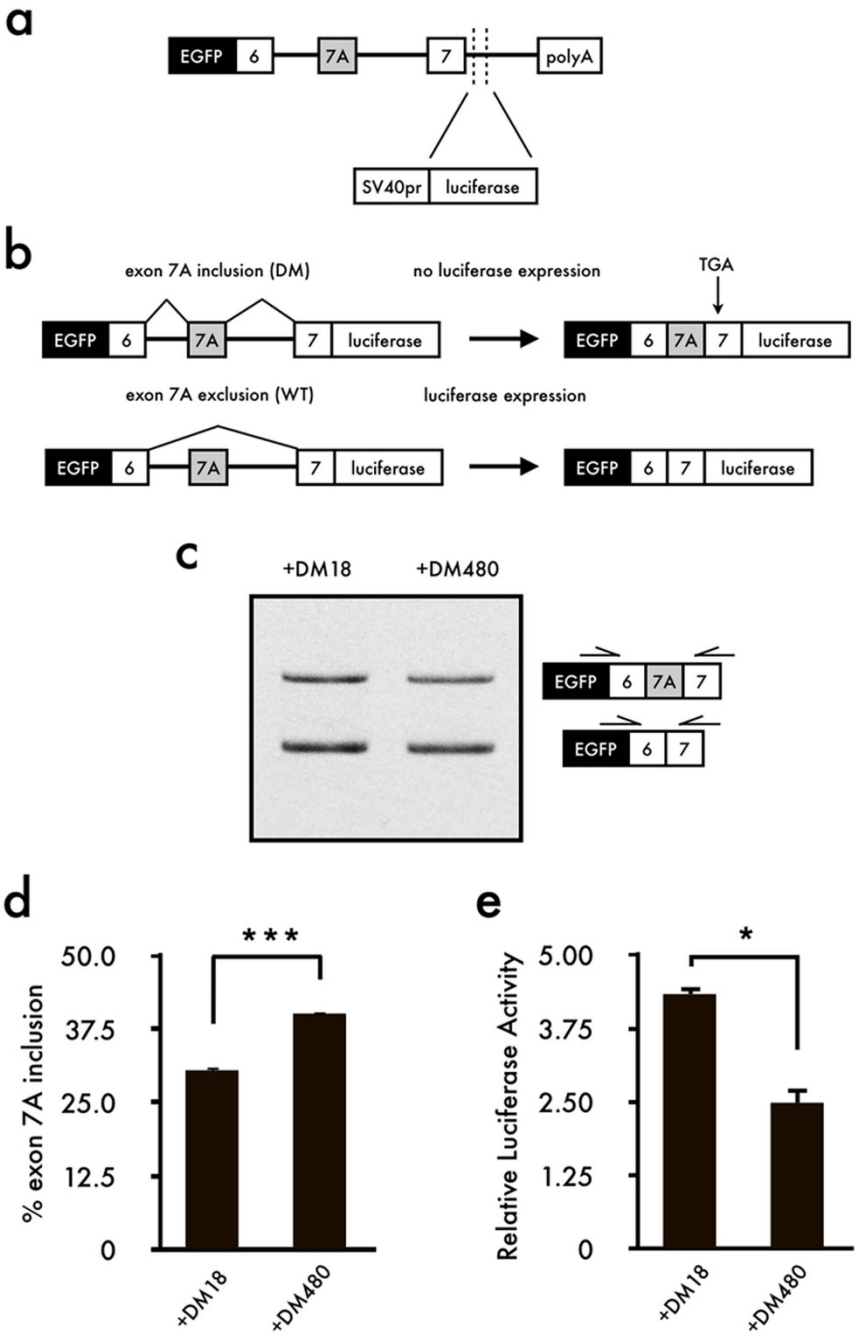

Figure 1 | Construction of the Clcn1-L reporter minigene and effect of triplet repeat expansion on the reporter vector Clcn1-L. (a) Schematic structure of the Clcn1-L minigene reporter. A genomic segment of mouse *Clcn1* containing exons 6 to 7 (including the intron) was sub-cloned downstream of EGFP in the pEGFP-C1 plasmid. Firefly luciferase was inserted in-frame with a correct *Clcn1* splicing pattern. (b) Schematic of how Clcn1-L functions. Exon 7A exclusion results in luciferase expression to detect correct *Clcn1* splicing. (c) Inclusion of *Clcn1* exon 7A increased upon expression of the expanded CUG repeat. (d) Bar charts show the quantified percentages of exon 7A inclusion (mean + SEM, n = 4). (e) Luciferase analysis showed that relative luciferase activity decreased upon expression of the expanded CUG repeat (mean + SEM, n = 3). The gel image was cropped around the region of interest and the samples (n = 4) were resolved in the same gel. Statistical significances were determined using *t*-tests (*$p < 0.05$, ***$p < 0.001$).

is also abnormally regulated in DM1[36,37], was not changed. It has been reported that *Serca1* is regulated by MBNL1[37,38] and CUGBP1[39] and that m-Titin is also regulated by MBNL1[37]. Furthermore, the expression of MBNL1 and CUGBP1 was not altered by treatment with manumycin A (Fig. S7). For these reasons, we conclude that manumycin A did not alter *Clcn1* splicing through effects on MBNL1 and CUGBP1. How, then, does manumycin A correct *Clcn1* missplicing? Manumycin A is an inhibitor of Ras farnesyltransferase, and it could inhibit Ras activity. In this study, we demonstrated that H-Ras knockdown reduced the inclusion of *Clcn1* exon7A, indicating that H-Ras is involved in a regulation of *Clcn1* splicing.

Ras proteins perform functional roles in a large number of biological processes, leading to changes in cell morphology, survival, apoptosis, and gene expression[40]. Because Ras is positioned as the central molecular switch of these biological outcomes, it must interact with a variety of downstream targets. Recent studies implicated the Ras signaling pathway in alternative splicing regulation[41]. Activation of the Ras-PI3-kinase-PKB/Akt pathway alters the

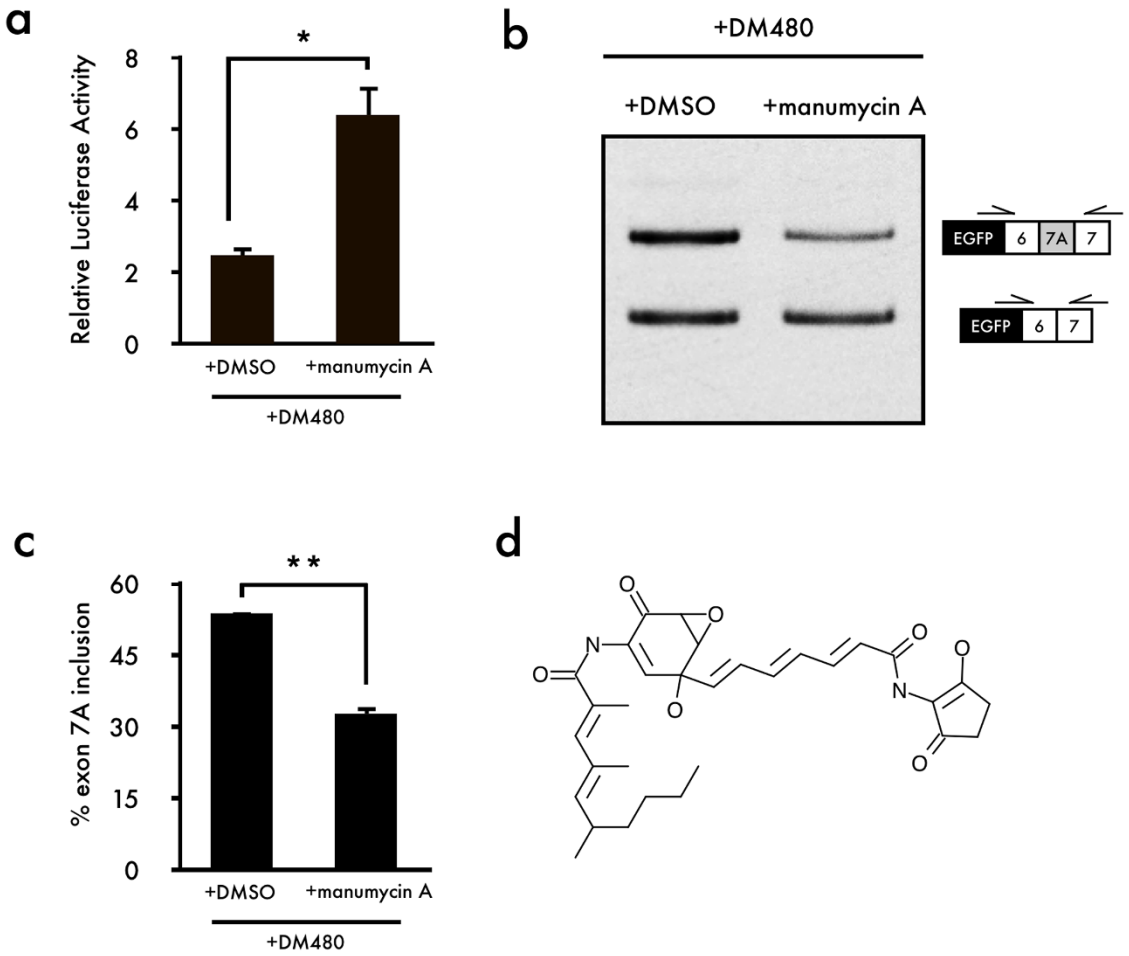

Figure 2 | Identification of small-molecule compounds that correct aberrant splicing of *Clcn1*. (a) A luciferase reporter assay showed that manumycin A corrected aberrant splicing in the presence of the expanded CUG repeat (mean + SEM, n = 3). (b) Cellular splicing analysis showed that manumycin A corrects aberrant splicing of *Clcn1*. (c) Quantification of the results shown in (b) (mean + SEM, n = 3). (d) Structure of manumycin A. The gel image was cropped around the region of interest and the samples (n = 3) were resolved in the same gel. Statistical significance was determined using *t*-tests (*$p < 0.05$, **$p < 0.01$).

phosphorylation level of the serine/arginine-rich (SR) proteins SF2/ASF and 9G8[42]. SR proteins are the best-characterized splicing regulatory factors, and it is well known that the activity of SR proteins is dependent on their phosphorylation level. Furthermore, the Ras-Raf-MEK-ERK pathway, another Ras pathway, is known to modulate alternative splicing[41,43].

Considering these studies, it is possible that manumycin A inhibits H-Ras activity and alters the H-Ras signaling pathway, resulting in correction of *Clcn1* splicing. Therefore, modulation of H-Ras signaling may influence a *trans*-acting factor other than MBNL1 and CUGBP1 and thereby contribute to *Clcn1* splicing. The effect of manumycin A is illustrated in Figure 5, although the question remains as to what kind of *trans*-acting factor is influenced by manumycin A and H-Ras. Importantly, manumycin A treatment showed reduced total amount of *Clcn1* mRNAs (with and without exon 7A) (Fig. 2b and Fig. 3c). Therefore, it is possible that manumycin A has another effect on either transcription or stability of *Clcn1* pre-mRNA.

A recent study revealed that oncogenic mutated H-Ras G12V inhibits muscle differentiation[44]. If H-Ras is involved in DM pathology, it may help us to better understand DM1. Interestingly, manumycin A only slightly changes the splicing of *Clcn1* in the absence of DM480 expression (Fig. S8). Furthermore, transfection of C2C12 cells with the expanded CUG repeat tended to increase H-Ras expression (Fig. S6). Considering the transfection efficiency of C2C12 cells, it is likely that the expanded CUG repeat could alter H-Ras expression. However, involvement of Ras in DM1 has not been reported thus far, and further studies are needed in the future.

DM1 is the most common muscular dystrophy in adults, affecting approximately one in every 8,000 individuals. However, there is as yet no cure for DM. In this study, we showed that manumycin A corrects aberrant splicing of *Clcn1* and revealed that H-Ras is involved in regulation of *Clcn1* splicing. We hope that further studies on manumycin A and H-Ras will lead to a novel therapy for DM1.

Methods

Plasmid construction. Clcn1-L is a luciferase reporter vector containing a genomic segment of mouse *Clcn1* (exon 6 to exon 7) and the firefly luciferase gene. Firefly luciferase (F-Luc) was amplified from the downstream of SV40 promoter of the pGL-3 promoter vector (Promega, Madison, WI, USA) by PCR using *PfuUltra* High-Fidelity DNA polymerase (STRATAGENE, La Jolla, CA, USA), generating a 1.7-kb product with the addition of a restriction site for *Sal*I and *Bam*HI. Therefore, this fragment did not contain SV40 promoter. The following primer pair was used: F-Luc forward, 5′-AAAGTCGACCCATGAAGACGCC-3′; and F-Luc reverse, 5′-CCGGATCCTTACACGGCGATCTT-3′. The fragment was inserted into the *Sal*I-*Bam*HI site of the Clcn1 minigene. The Clcn1 minigene contains PCR-amplified fragments of mouse *Clcn1* (exon 6 to exon 7) and has been described previously[22]. The nucleotide sequences of the DNA inserts and reading frame were confirmed to be correct by sequencing. DM18 and DM480 contain a fragment of the 3′ region of *DMPK* with CTG18 or interrupted CTG480 repeats, respectively, and have been described previously[23].

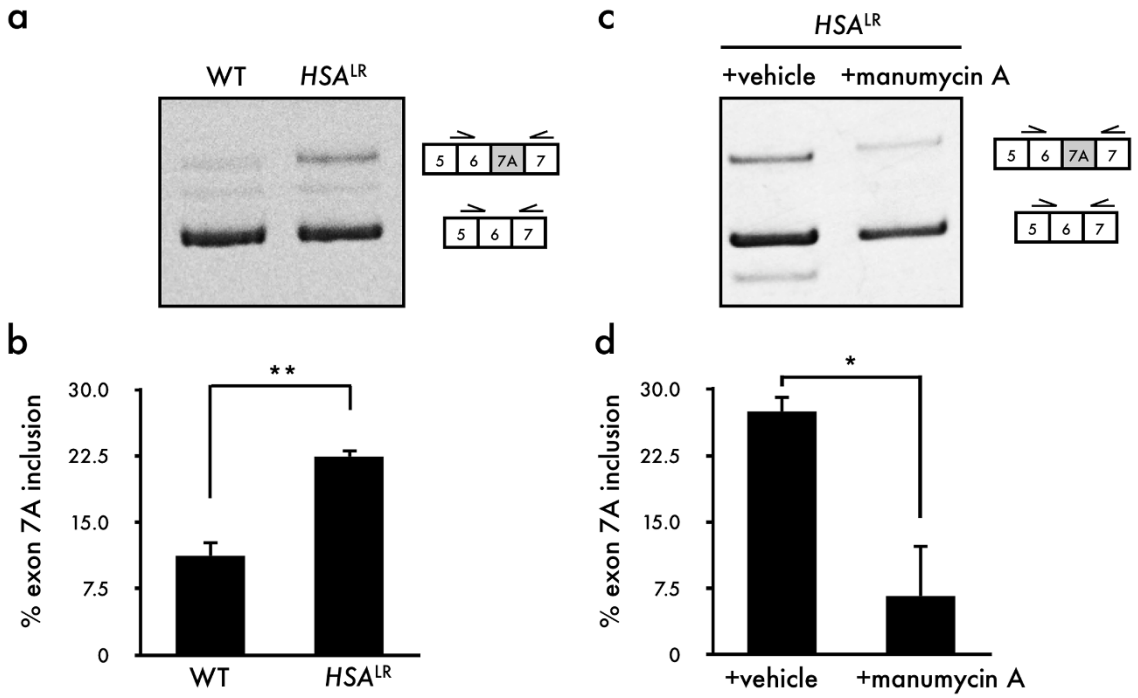

Figure 3 | Manumycin A corrects aberrant splicing of *Clcn1* in *HSA*^{LR} DM1 model mice. (a) RT-PCR analysis showed increased inclusion of *Clcn1* exon 7A in *HSA*^{LR} mouse. (b) Quantification of the results shown in (a) (mean + SEM, n = 3). (c) RT-PCR analysis showed reduced inclusion of *Clcn1* exon 7A 5 days after injection of manumycin A into TA muscles. (d) Quantification of the results shown in (a) (Mean + SEM, n = 3). The gel image was cropped around the region of interest and the samples (n = 3) were resolved in the same gel. Statistical significance was determined using *t*-tests (*$p < 0.05$, **$p < 0.01$).

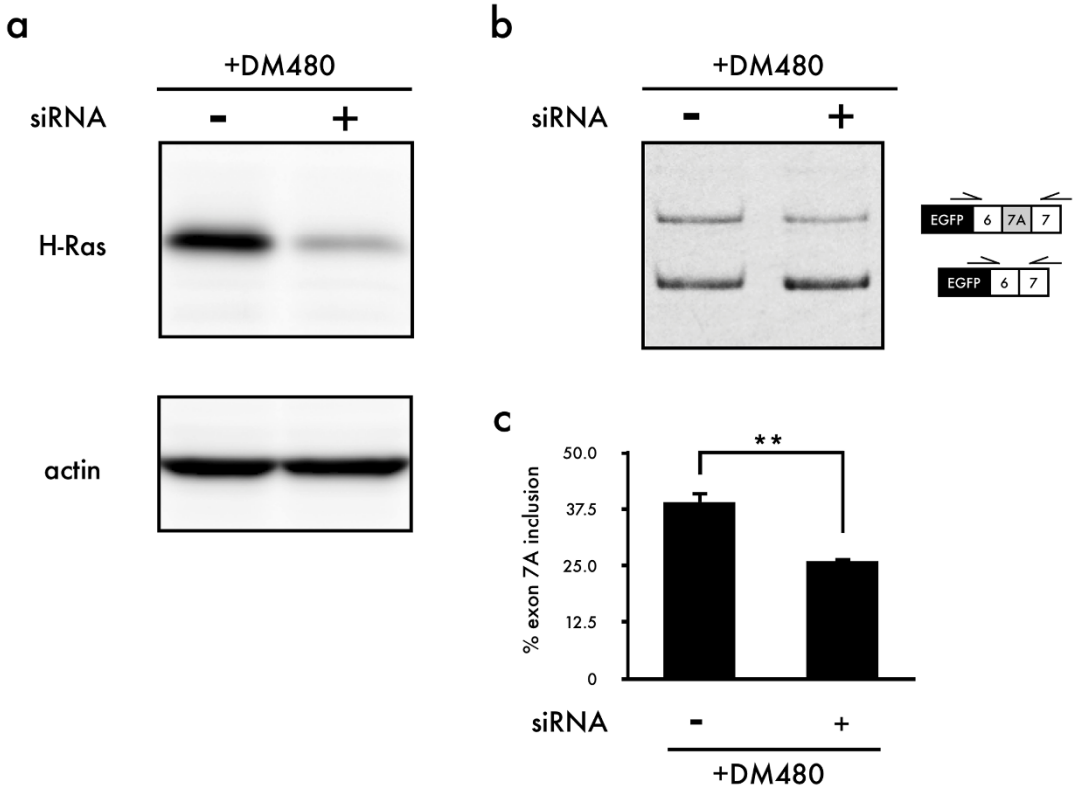

Figure 4 | The knockdown of H-Ras corrects aberrant splicing of *Clcn1* in the presence of the expanded CUG repeat. (a) Representative result of Western blot analysis of H-Ras in C2C12 cells. (b) Results of cellular splicing assays using Clcn1-L minigene, DM480 and siRNA. (c) Quantification of the results shown in (b) (mean + SEM, n = 3). The gel and blot image were cropped around the region of interest and the samples (n = 3) were resolved in the same gel or blot. Statistical significance was determined using *t*-tests (**$p < 0.01$).

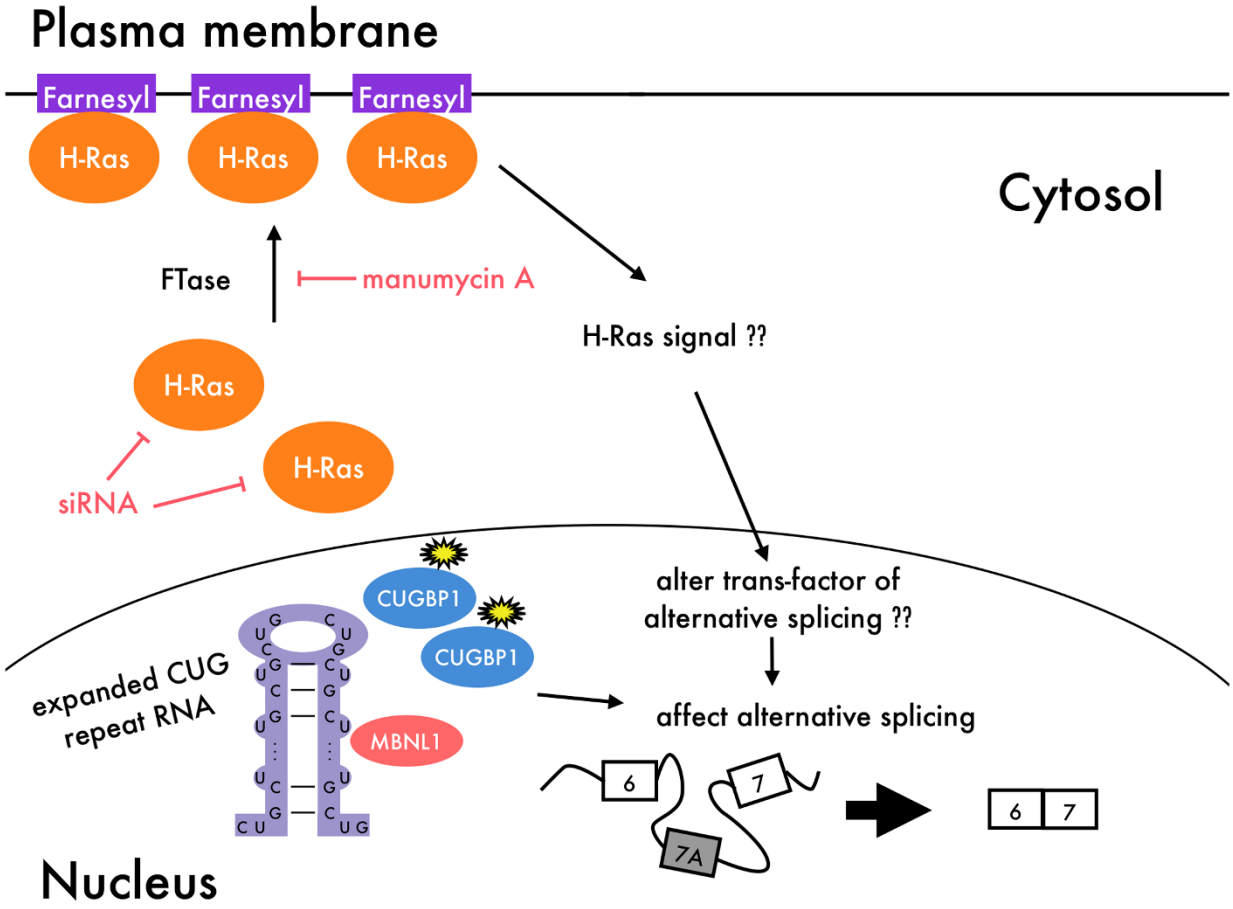

Figure 5 | Model of how manumycin A affects *Clcn1* splicing. In DM1, it is known that expanded CUG repeat RNA transcripts trap MBNL1 and up-regulate CUGBP1, resulting in aberrant regulation of alternative splicing. However, manumycin A does not affect the expression of these proteins. Manumycin A might act as a Ras farnesyltransferase inhibitor, thereby altering H-Ras signaling. This could in turn result in alteration of a *trans*-acting factor involved in alternative splicing other than MBNL1 and CUGBP1.

Cell culture and transfection. C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) fetal bovine serum and then incubated at 37°C with 5% CO_2. For the minigene and luciferase reporter assays, C2C12 cells were transfected with plasmids for expression of a minigene with toxic RNA transcripts using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection was conducted according to the manufacturer's protocol. Cells were cultured in 24- and 96-well plates for the minigene and luciferase reporter assays, respectively.

Luciferase reporter assay and drug screening. Culture, transfection, cell harvesting, and luciferase activity measurements were performed according to the standard methods of the Dual-Glo Luciferase Assay System (Promega). Clcn1-L and DM480 were co-expressed in cells. Plasmid pRL (Promega), which contains the sea pansy luciferase gene, was co-transfected as an internal control for normalization of transfection efficiency. In all experiments, luciferase activity was measured 48 h after transfection and was assayed using the Dual-Glo Luciferase Assay System (Promega). Firefly and sea pansy luciferase activities were measured using the Centro LB 960 (Berthold, Bad Wildbad, Germany), and the value of each sample was calculated as light units of firefly luciferase per light unit of sea pansy. The ICCB Known Bioactives Library (Enzo Life Sciences, Farmingdale, NY, USA) was referenced for drug screening. Chemical compounds were added 24 h after transfection at 0.1% (v/v), and luciferase activity was measured 24 h after the addition of chemical compounds.

Administration of manumycin A to DM mice. HSA^{LR} transgenic mice were used for animal experiments and have been described previously[45]. These mice express human skeletal actin mRNA, with approximately 250 CUG repeats in the 3′-UTR. Manumycin A was diluted to a final concentration of 75 ng/μl in saline containig 0.1% DMSO. Manumycin A (40 μl, 3.0 μg) or vehicle (0.1% DMSO in saline) was injected into the TA muscles of opposite limbs. Mice were killed 5 days after injection, and TA muscle was obtained for splicing analysis. All experiments were conducted according to the Regulations for Animal Experimentation at the University of Tokyo (Tokyo, Japan).

Identification of Clcn1 splice variants. Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA synthesis was performed using a Prime-Script First Strand cDNA Synthesis Kit (TAKARA BIO, Otsu, Japan) with an oligo dT primer. The Clcn1-L minigene fragments were amplified by PCR (24–27 cycles) with the following primer pair: Clcn1-L forward, 5′-CATGGTCCTGCTGGAGTTCGTG-3′; and Clcn-L reverse, 5′-CTCCAAGTGGTGTTCCAAAACAGC-3′. To detect endogenous *Clcn1* fragments, PCR amplification (32-34 cycles) was carried out with the following primer pair: *Clcn1* forward, 5′-GCTGCTGTCCTCAGCAAGTT-3′; and *Clcn1* reverse, 5′-CTGAATGTGGCTGCAAAGAA-3′. PCR products were resolved on 8% polyacrylamide gels and stained with ethidium bromide. Band intensities were quantified using an LAS-3000 instrument and Multigauge software (FUJIFILM, Tokyo, Japan). The ratio of exon 7A inclusion in *Clcn1* was calculated as (7A inclusion)/(7A inclusion + 7A exclusion) × 100.

RNA interference. An siRNA specific for H-Ras (H-Ras siRNA) and a negative control siRNA (MISSION siRNA Universal Negative Control) were purchased from Sigma-Aldrich. The siRNA target sequences were as follows: mouse H-Ras siRNA sense, 5′- GUUGCAUCACAGUAAAUUAdTdT-3′; and mouse H-Ras siRNA antisense, 5′- UAAUUUACUGUGAUGCAACdTdT-3′. The efficacy of the RNAi-mediated knockdown of endogenous H-Ras and actin expression was determined by Western blot analysis. Antibodies specific for H-Ras (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) and actin (A2066; Sigma-Aldrich) were used.

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Author contributions

K.O., Y.O. and S.I. designed the experiments; K.O. performed all experiments; Y.O. assisted with the construction of the reporter vector; S.S. assisted with the interpretation of the results; M.P.T. prepared the mice; S.T. prepared the small chemical compound library; N.I. supported the project; S.I. supervised the work; and K.O. analyzed the data and wrote the manuscript.

Additional information

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