Supplemental Material for:

Intracellular distribution and nuclear activity of antisense oligonucleotides after unassisted uptake in myoblasts and differentiated myotubes *in vitro*

Anchel González-Barriga[*,†], Bram Nillessen[*], Julia Kranzen[*], Ingeborg D. G. van Kessel[*], Huib J. E. Croes[*], Begoña Aguilera[†], Peter C. de Visser[†], Nicole A. Datson[†], Susan A. M. Mulders[†], Judith C. T. van Deutekom[†], Bé Wieringa[*] and Derick G. Wansink[*,#]

* Radboud Institute for Molecular Life Sciences, Radboud university medical center, Department of Cell Biology, Nijmegen, The Netherlands

† BioMarin Nederland B.V., Leiden, The Netherlands

[#] Author for correspondence: rick.wansink@radboudumc.nl

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Supplementary Table S1. Primers and TaqMan® assays used in this study.

Name	Sequence (5' -> 3') or assay ID*
mDmd e22-e24 Fw	ATCCAGCAGTCAGAAAGCAAA
mDmd e22-e24 Rv	CAGCCATCCATTTCTGTAAGG
hDMPK e1-e2 Fw	ACTGGCCCAGGACAAGTACG
hDMPK e1-e2 Rv	CCTCCTTAAGCCTCACCACG
hDMPK e15 (5′) Fw	AGAACTGTCTTCGACTCCGGG
hDMPK e15 (5′) Rv	TCGGAGCGGTTGTGAACTG
hDMPK e15 (3′) Fw	TGCCTGCTTACTCGGGAAATT
hDMPK e15 (3′) Rv	GAGCAGCGCAAGTGAGGAG
mActb Fw	GCTCTGGCTCCTAGCACCAT
mActb Rv	GCCACCGATCCACACAGAGT
mGapdh Fw	GTCGGTGTGAACGGATTTG
mGapdh Rv	GAACATGTAGACCATGTAGTTG
mHprt1 Fw	CCTAAGATGAGCGCAAGTTGAA
mHprt1 Rv	CCACAGGACTAGAACACCTGCTAA
Myh1 (TaqMan)	Mm01332489_m1*
Mylfp (TaqMan)	Mm00443940_m1*
Cav3 (TaqMan)	Mm01182632_m1*
Aqp1 (TaqMan)	Mm01326466_m1*

*Assay ID according to Applied Biosystems (Thermo Fisher Scientific) denomination.

Primers for *Gapdh* were described earlier (Fu et al. (2007) J. Biomed. Mater. Res. A, 83, 770-778).

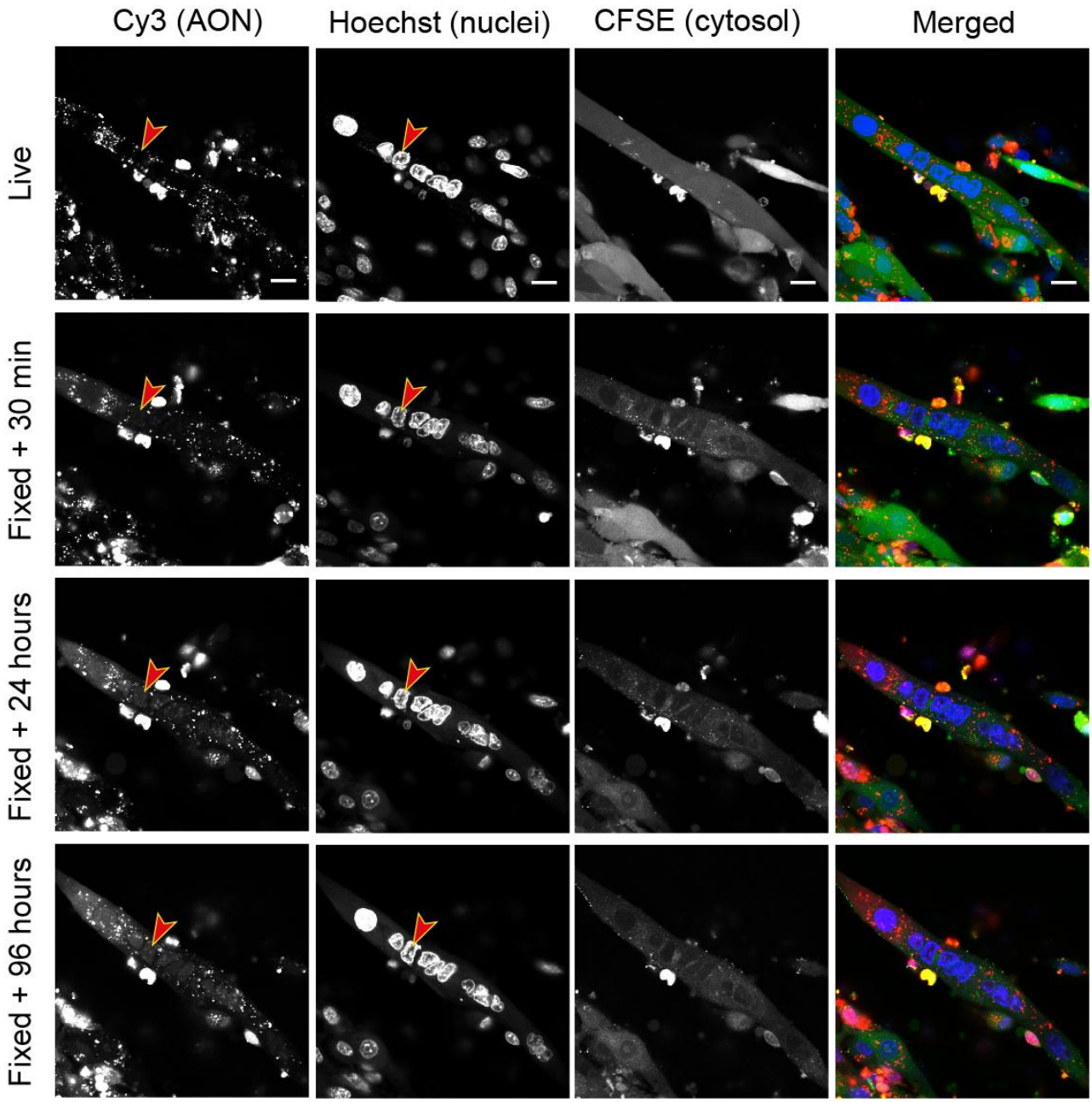

Figure S1. Intracellular AON distribution changes in time after regular formaldehyde fixation.

DM500 myotubes grown in the presence of 500 nM Cy3-DMD23-OMePS for seven days were imaged live (compare Fig. 3) and at different time points following formaldehyde fixation, as indicated. Following fixation, AON signal progressively diffused into nuclei (e.g., arrowhead) when cells were kept in PBS. Scale bars indicate 20 µm.

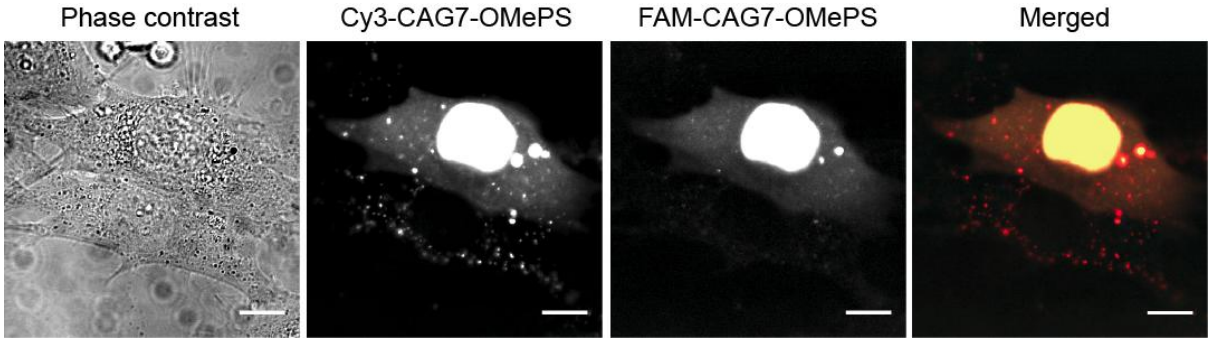

Figure S2. Representative AON localization patterns after PEI-mediated transfection.

DM500 myoblasts were transfected using PEI with a mixture of Cy3- and FAM-conjugated CAG7-OMePS (each 200 nM) and imaged after two days. The image shows two cells with different uptake patterns. The upper one displays strong nuclear signal (traditionally considered positive for transfection), whereas the lower one presents only faint vesicular staining and essentially lacks nuclear signal (traditionally considered negative for transfection). Scale bars indicate 10 µm.

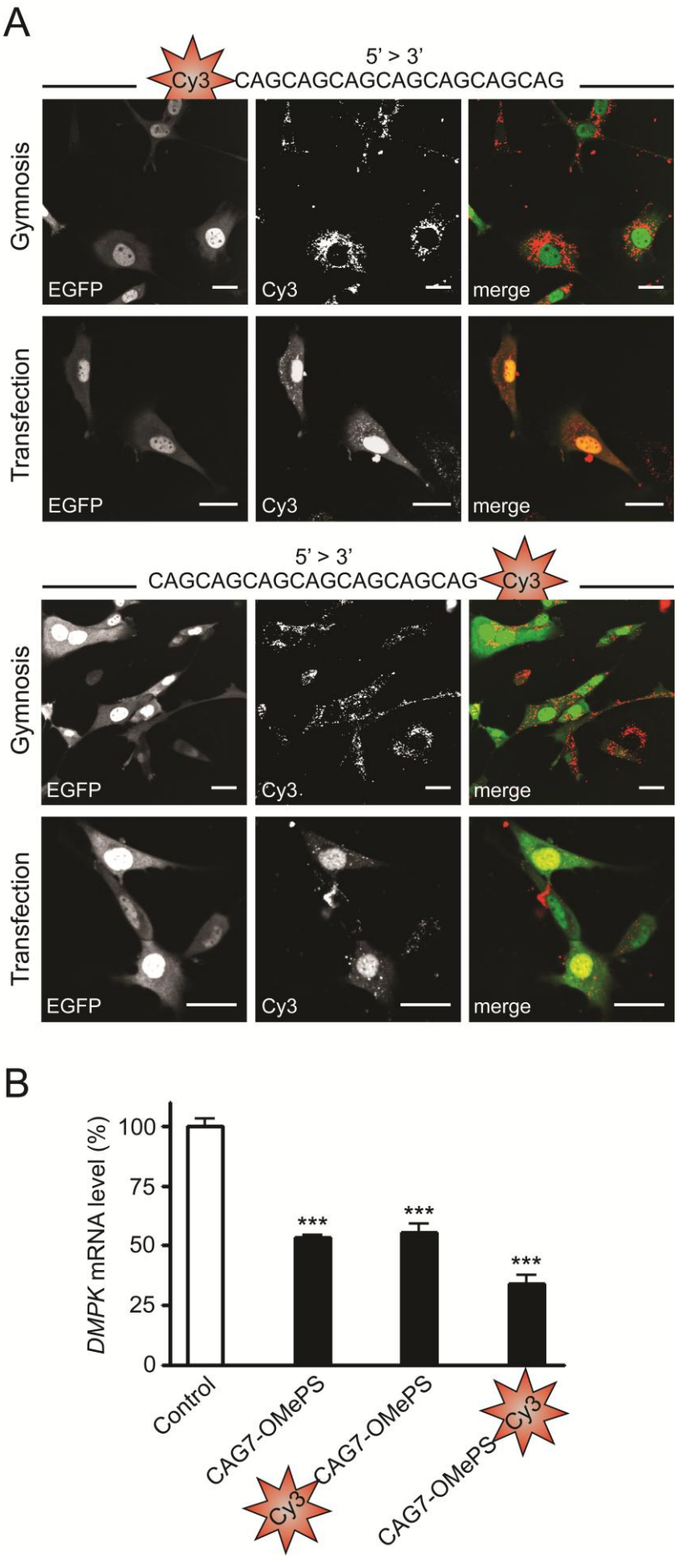
A
5' > 3'
Cy3
CAGCAGCAGCAGCAGCAGCAG
Gymnosis
Transfection
EGFP
Cy3
merge
5' > 3'
CAGCAGCAGCAGCAGCAGCAG
Cy3
B
DMPK mRNA level (%)
100
75
50
25
0
***
***
***
Control
CAG7-OMePS
CAG7-OMePS
Cy3
CAG7-OMePS
Cy3

Figure S3. No effect of the fluorophore position on gymnosis.

(A) Localization of CAG7-OMePS, Cy3 conjugated at either its 5' or 3' end, after 48 hours of gymnosis or PEI transfection (500 nM) in DM500 EGFP-Mbnl1 myoblasts. Scale bars indicate 25 µm. **(B)** Quantification of expression of expanded *DMPK* transcripts after 48 hours of gymnosis with CAG7-OMePS Cy3-conjugated at either its 5' or 3' end (500 nM). Data was analyzed by 1-way ANOVA followed by a Bonferroni's Multiple Comparison Test (***: $p<0.001$).

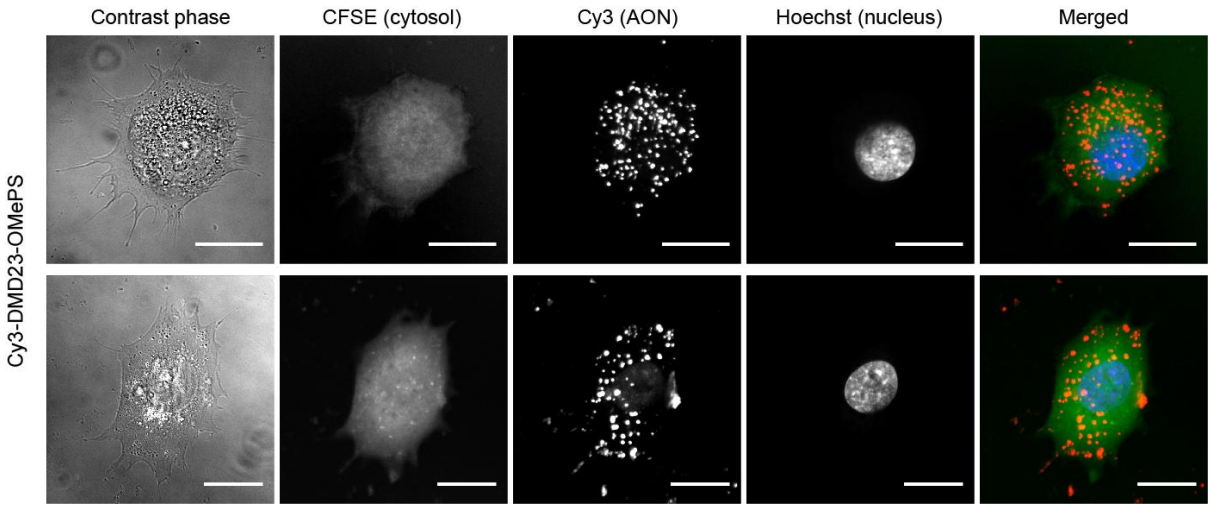

Figure S4. Examples of cellular distribution patterns after gymnosis of Cy3-DMD23-OMePS.

DM500 myoblasts were imaged after 24 hours gymnosis incubation with Cy3-DMD23-OMePS (500 nM). A spotted distribution in the nucleus was observed in some cells (top row) and, less frequently, AON signal appeared diffuse throughout the nucleus (bottom row). Scale bars indicate 20 µm.

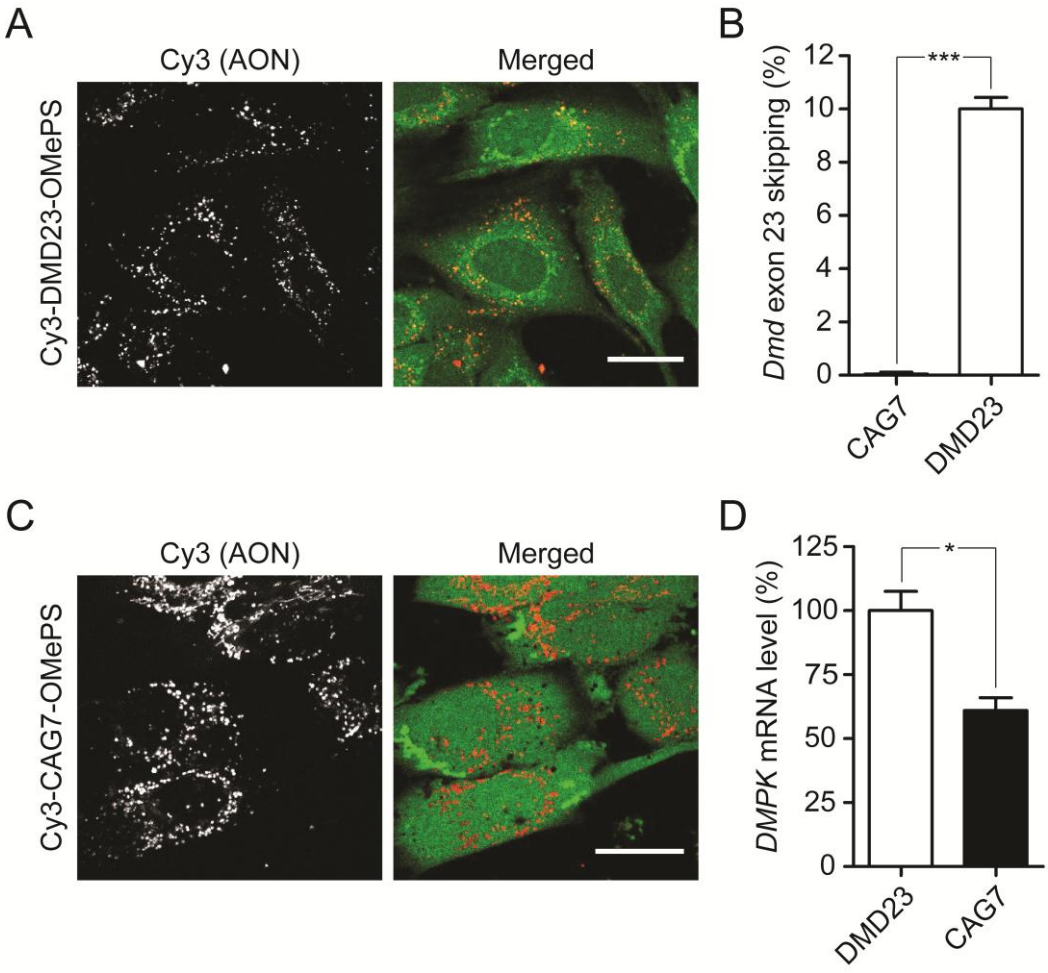

Figure S5. Cellular distribution and nuclear activity of (Cy3-)DMD23-OMePS and (Cy3-)CAG7-OMePS after gymnosis in mouse C2C12 myoblasts and human DM1 myoblasts.

Mouse C2C12 and human DM1 myoblasts were cultured in the presence of DMD23-OMePS or CAG7-OMePS (or their Cy3-conjugated analogs; 500 nM) according to the protocol depicted in Fig. 6B. **(A)** Cellular uptake of Cy3-DMD23-OMePS by C2C12 myoblasts after 48 hours of gymnosis. CSFE (green) was used to visualize the contour of each cell and its nucleus. **(B)** Effect on *Dmd* exon 23 skipping by DMD23-OMePS in C2C12 myotubes after nine days of gymnosis. **(C)** Cellular uptake of Cy3-CAG7-OMePS by DM1 myoblasts after 48 hours of gymnosis. **(D)** Effect on *DMPK* mRNA levels by CAG7-OMePS in DM1 myotubes after nine days of gymnosis. Significance was assessed by t-test analysis (*: $p<0.05$; ***: $p<0.001$). Scale bars indicate 25 µm.

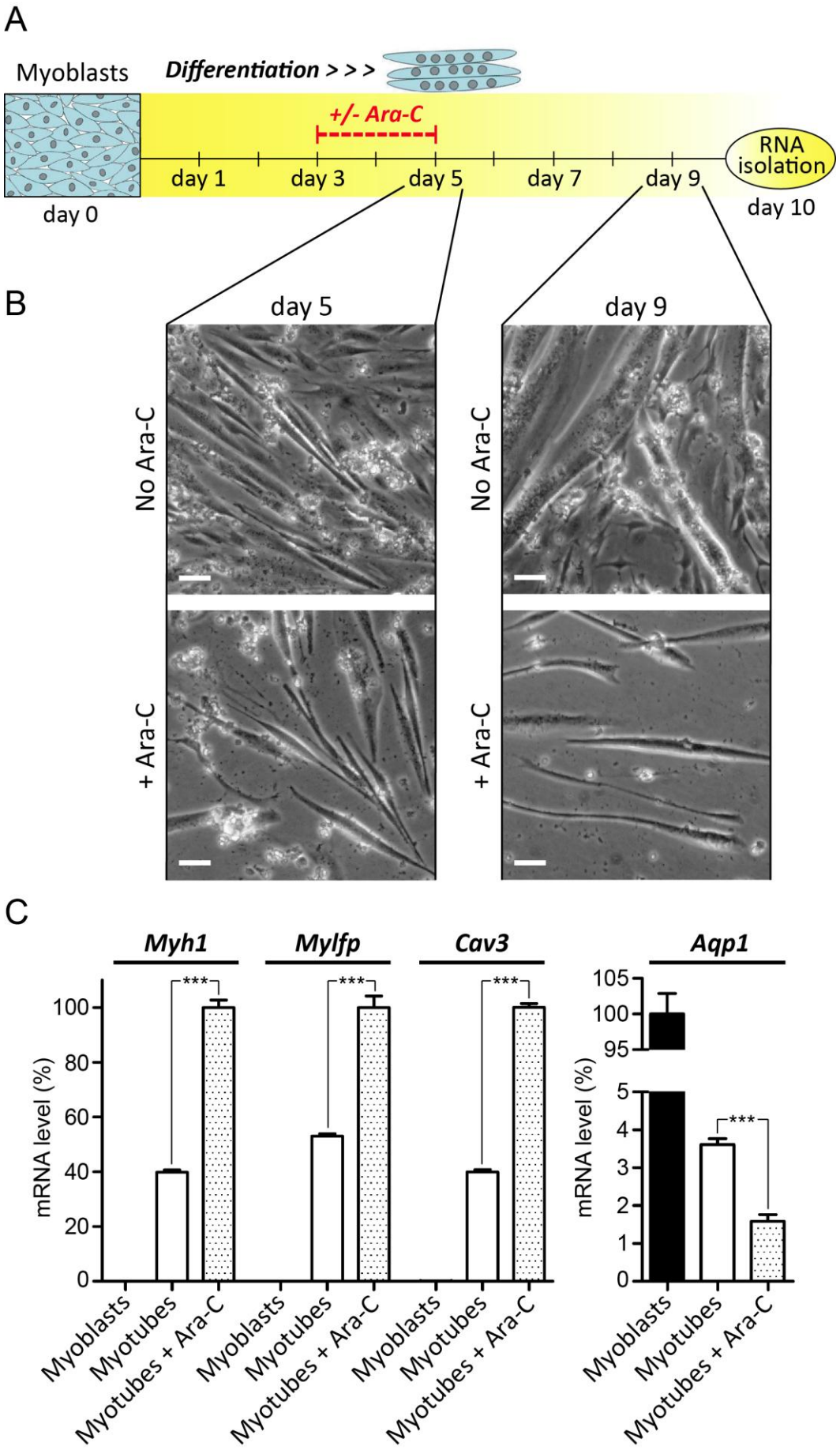
A
Myoblasts
Differentiation > > >
+/- Ara-C
RNA isolation
day 0
day 1
day 3
day 5
day 7
day 9
day 10
B
day 5
day 9
No Ara-C
+ Ara-C
C
Myh1
Mylfp
Cav3
Aqp1
mRNA level (%)
***
Myoblasts
Myotubes
Myotubes + Ara-C

Figure S6. Validation of Ara-C treatment.

(A) Scheme illustrating Ara-C treatment on day 3 to 5 of myogenic differentiation. **(B)** Representative images of cell cultures acquired at the end of the Ara-C incubation period and four days later. Scale bars indicate 50 µm. **(C)** Comparison of myogenic marker expression at day 10 of myogenesis (with/without Ara-C treatment). RNA from proliferating myoblasts was taken along as a control (n=6). *Myh1*, *Mylfp* and *Cav3* expression was induced during myogenesis and was significantly enriched after Ara-C treatment. *Aqp1* was mainly expressed in myoblasts, silenced during myogenesis and increasingly lost after Ara-C treatment. For each gene, mRNA levels of untreated and Ara-C-treated myotubes were compared by unpaired t-tests (***: $p<0.001$).

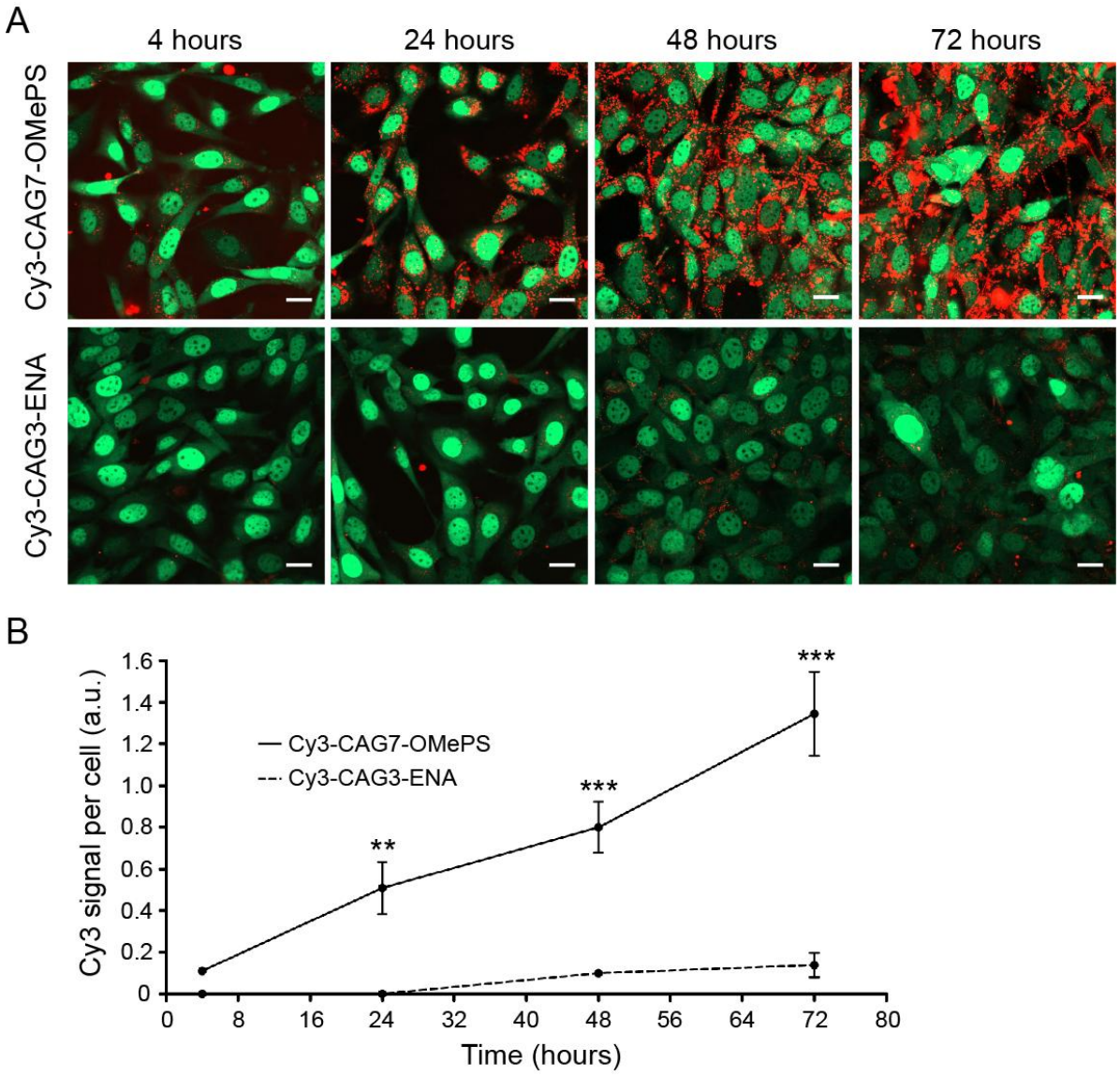

Figure S7. Gymnosis of Cy3-CAG7-OMePS versus Cy3-CAG3-ENA.

DM500 EGFP-Mbnl1 myoblasts were cultured for three days in the presence of Cy3-conjugated AONs (200 nM). The behaviour of Cy3-CAG7-OMePS (compare Fig. 1) was compared with that of Cy3-CAG3-ENA, a much shorter repeat AON carrying an ethylene-bridged nucleic acid phosphate backbone. **(A)** Confocal images showing intracellular localization of AONs (red). Cy3-CAG7-OMePS quickly accumulated in cytoplasmic vesicles. In contrast, Cy3-CAG3-ENA was poorly taken up. Scale bars indicate 20 µm. **(B)** Quantification of red fluorescence (n=3; 30 cells per experiment). Data was analyzed by 2-way ANOVA (**: $p<0.01$, ***: $p<0.001$).

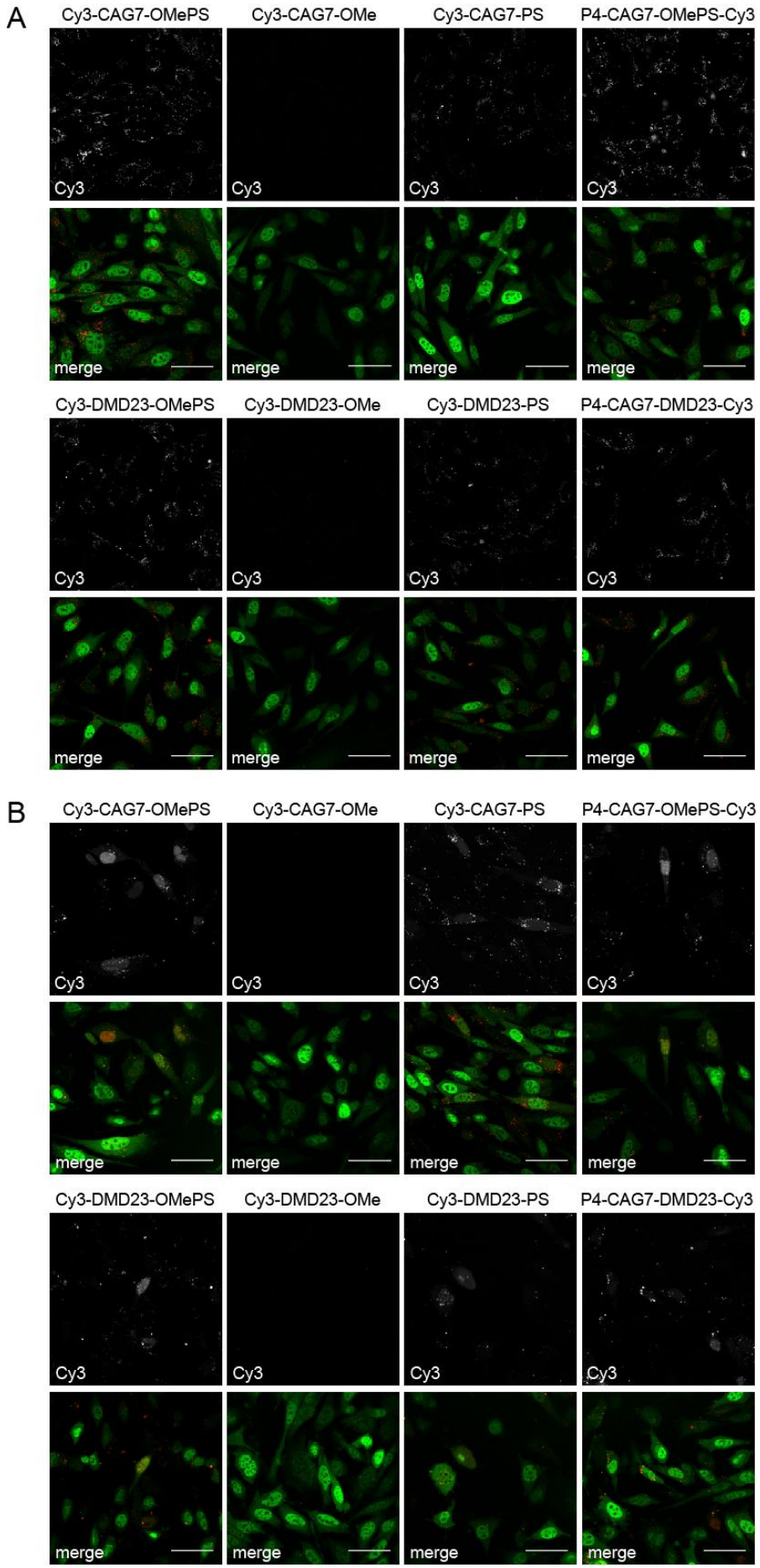
A
Cy3-CAG7-OMePS
Cy3-CAG7-OMe
Cy3-CAG7-PS
P4-CAG7-OMePS-Cy3
Cy3
merge
Cy3-DMD23-OMePS
Cy3-DMD23-OMe
Cy3-DMD23-PS
P4-CAG7-DMD23-Cy3
B
Cy3-CAG7-OMePS
Cy3-CAG7-OMe
Cy3-CAG7-PS
P4-CAG7-OMePS-Cy3
Cy3-DMD23-OMePS
Cy3-DMD23-OMe
Cy3-DMD23-PS
P4-CAG7-DMD23-Cy3

Figure S8. Representative images used for quantification of Cy3 signal in nucleus and cytoplasm (see Fig. 6)

Images were acquired below saturation limits in the same microscopic session after treatment with the indicated AONs **(A)** during 48 hours of gymnosis or **(B)** PE-mediated transfection. Merged images are a composite of Cy3 and EGFP channels. Scale bars indicate 50 µm.