Supplemental data

iPSC-derived functional human neuromuscular junctions model the pathophysiology of neuromuscular diseases

Authors

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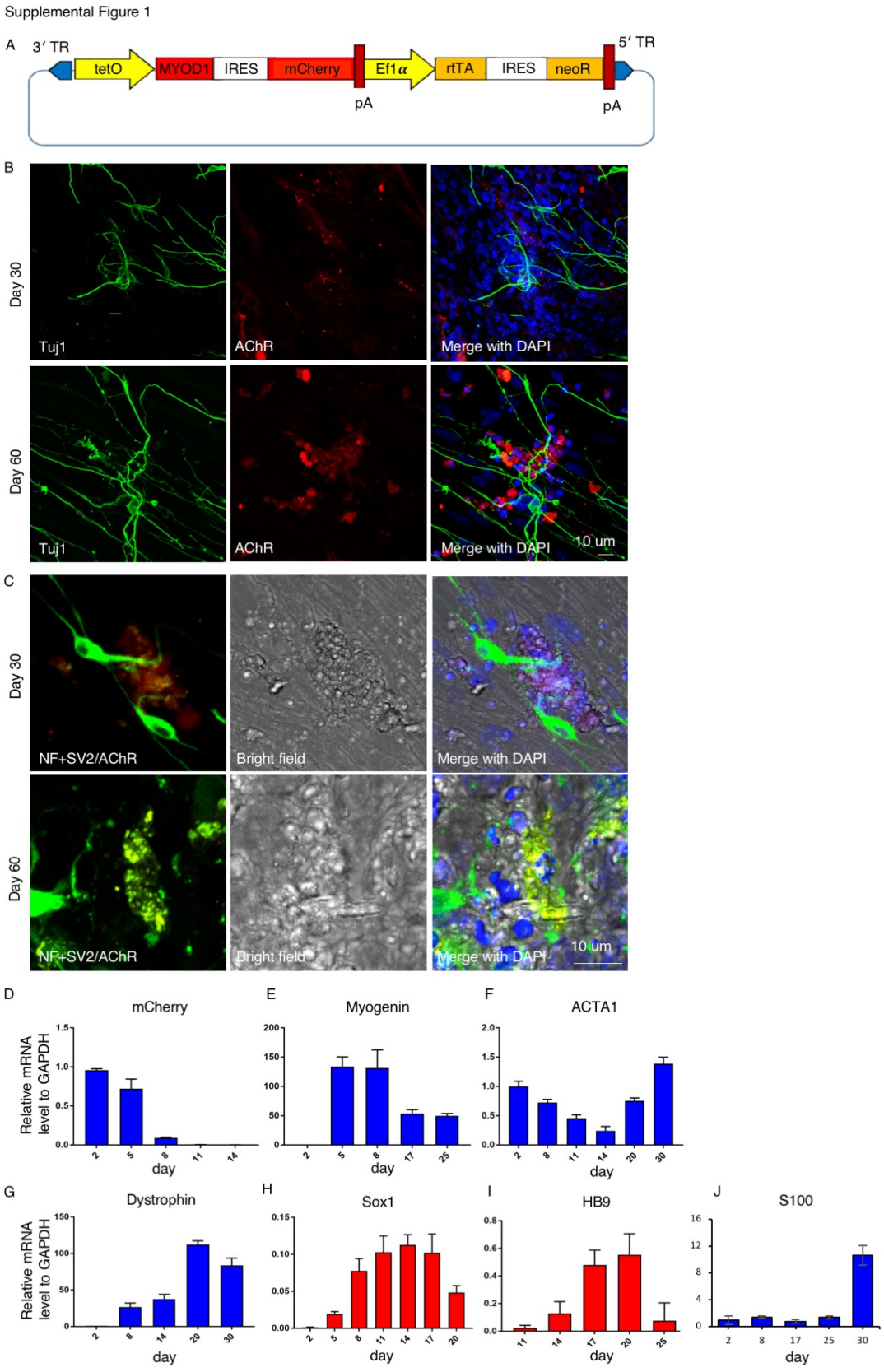
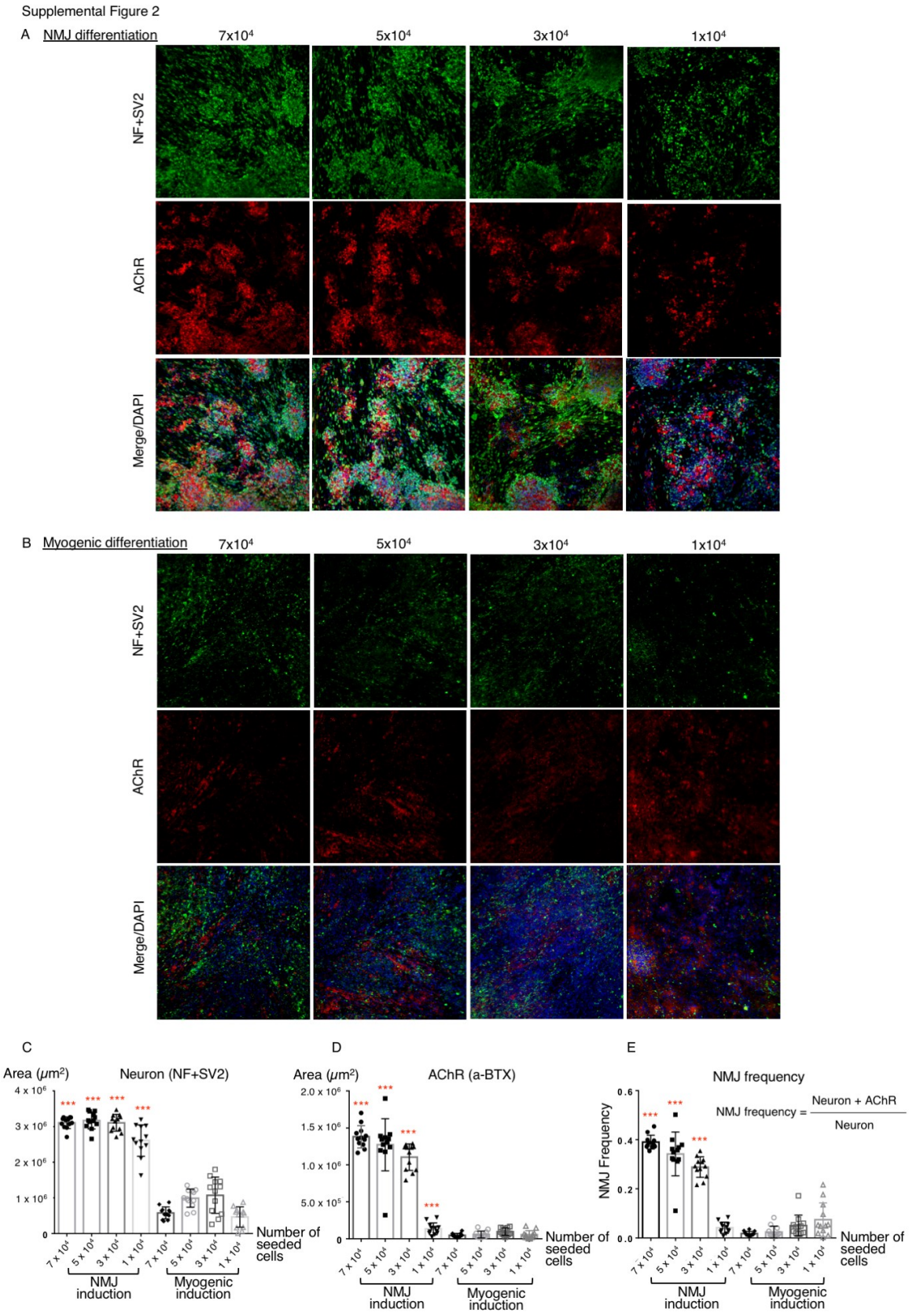
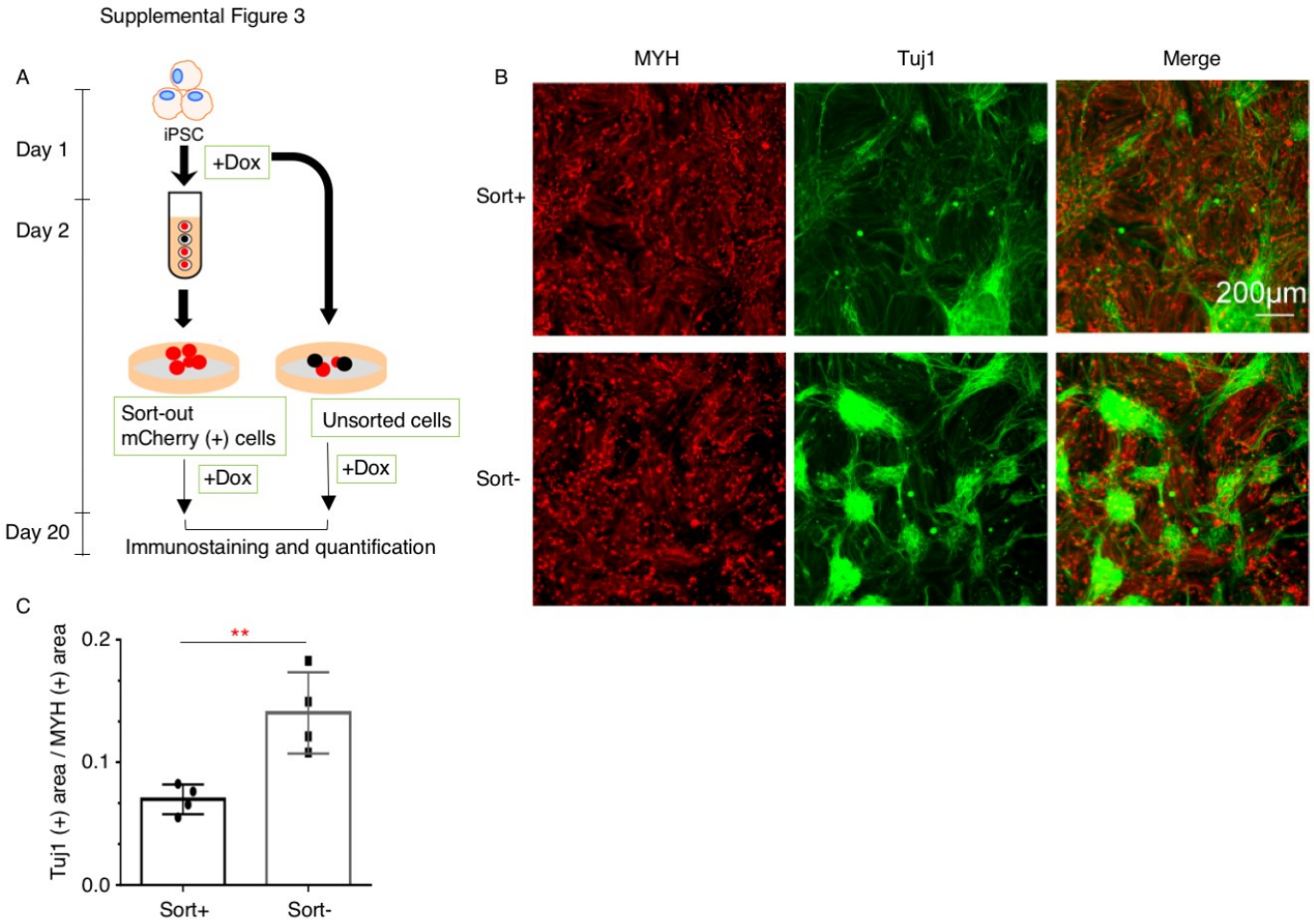


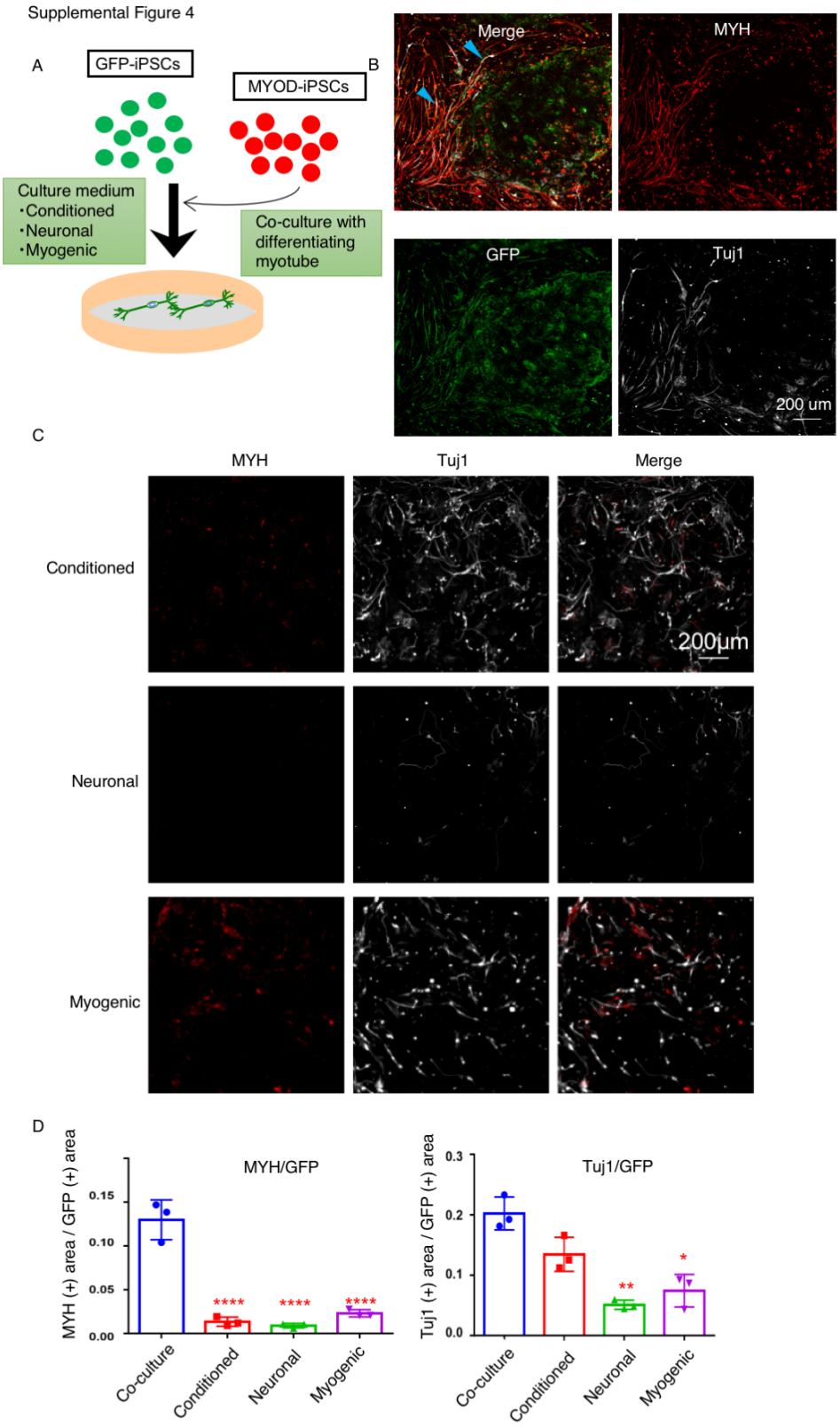
Supplemental Figure 1. (A) The expression construct of MYOD1 with doxycycline (Dox)-inducible vector (related to Figure 1). (**B**) DAPI staining shows synaptic nuclei in the NMJ culture (related to Figure 2B). **(C)** Bright field images show myotube morphology in the culture (related to Figure 2C). **(D-J)** Gene expression profiles including myogenic markers (E-G), neural markers (H, I) and a Schwann cell marker (J) expressed by 201B7^{MYOD} during NMJ formation. Myogenin indicates myoblasts; ACTA1 and Dystrophin indicate myotubes**;** SOX1 indicates neural stem cells; HB9 indicates motor neurons; and S100 indicates Schwann cells. Data are means ± SEM, n=3.



Supplemental Figure 2. Effect of conditions for NMJ formation or myogenic induction on NMJ differentiation. Quantitative immunocytochemistry analysis of areas of neurons and AChRs and of NMJ frequencies. **(A** and **B)** Representative images of NMJ differentiation and myogenic differentiation at various cell numbers. **(C)** The areas of neurons (NF+SV2) **(D)** the areas of AChRs labeled by a-BTX, and **(E)** the NMJ frequencies in NMJ cultures. ***: $p<0.001$ (unpaired Student's two-sided t-test relative to the same cell number). Data are means ± SEM, n=12. Each dot represents a biologically independent sample.

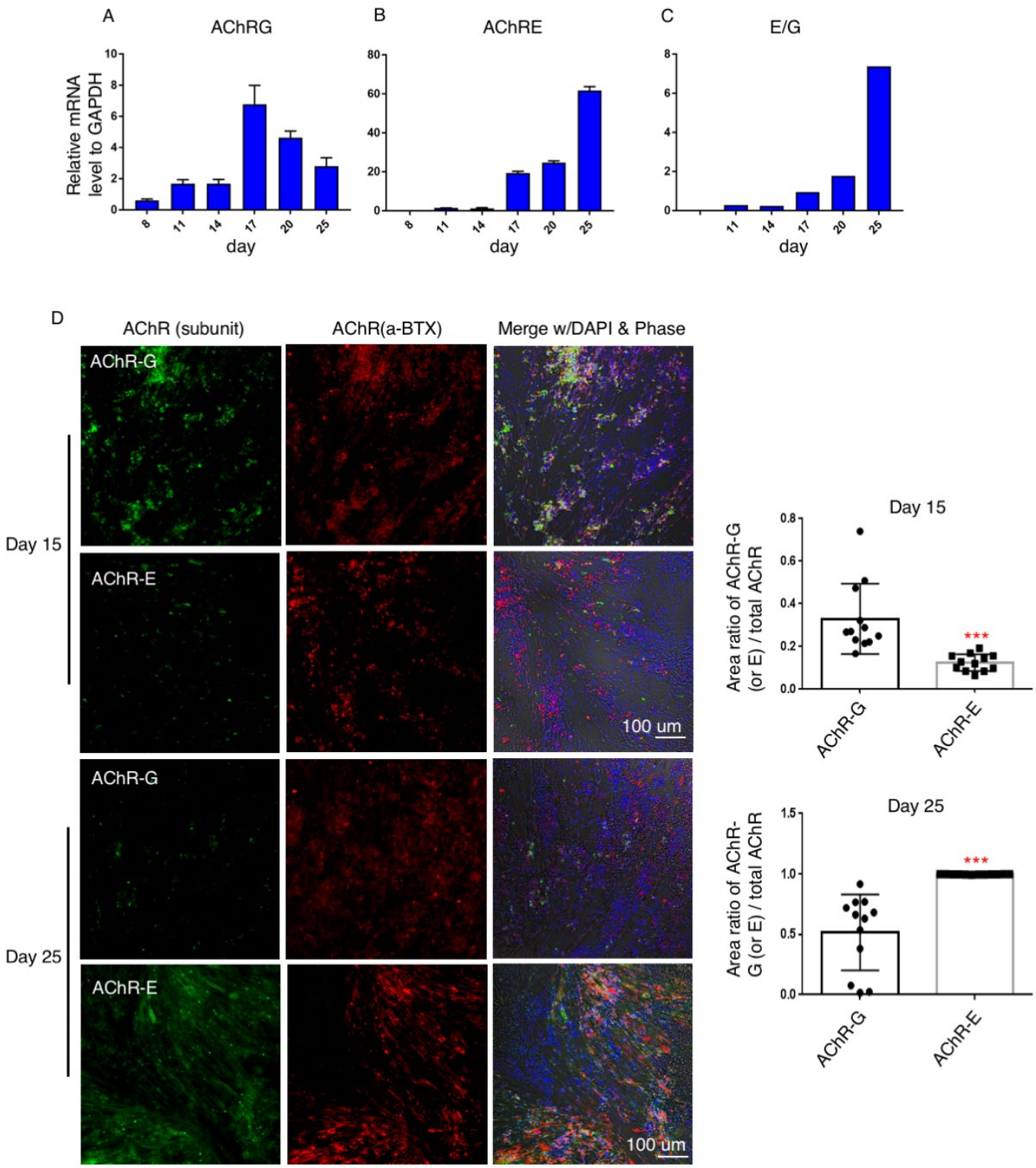


Supplemental Figure 3. Effect of cell sorting and Dox administration on NMJ differentiation. (A) Scheme of the experiment. Cell sorting was performed on differentiation day 2. 201B7^{MYOD} cells were cultured in myogenic differentiation medium with Dox. **(B)** Representative images for each experimental condition. **(C)** The ratio of the area of Tuj1+ neurons to the area of MHC+ myotubes was quantified by immunocytochemistry on day 20. n=4. **: $p<0.01$ (unpaired Student's two-sided t-test relative to Sort+).



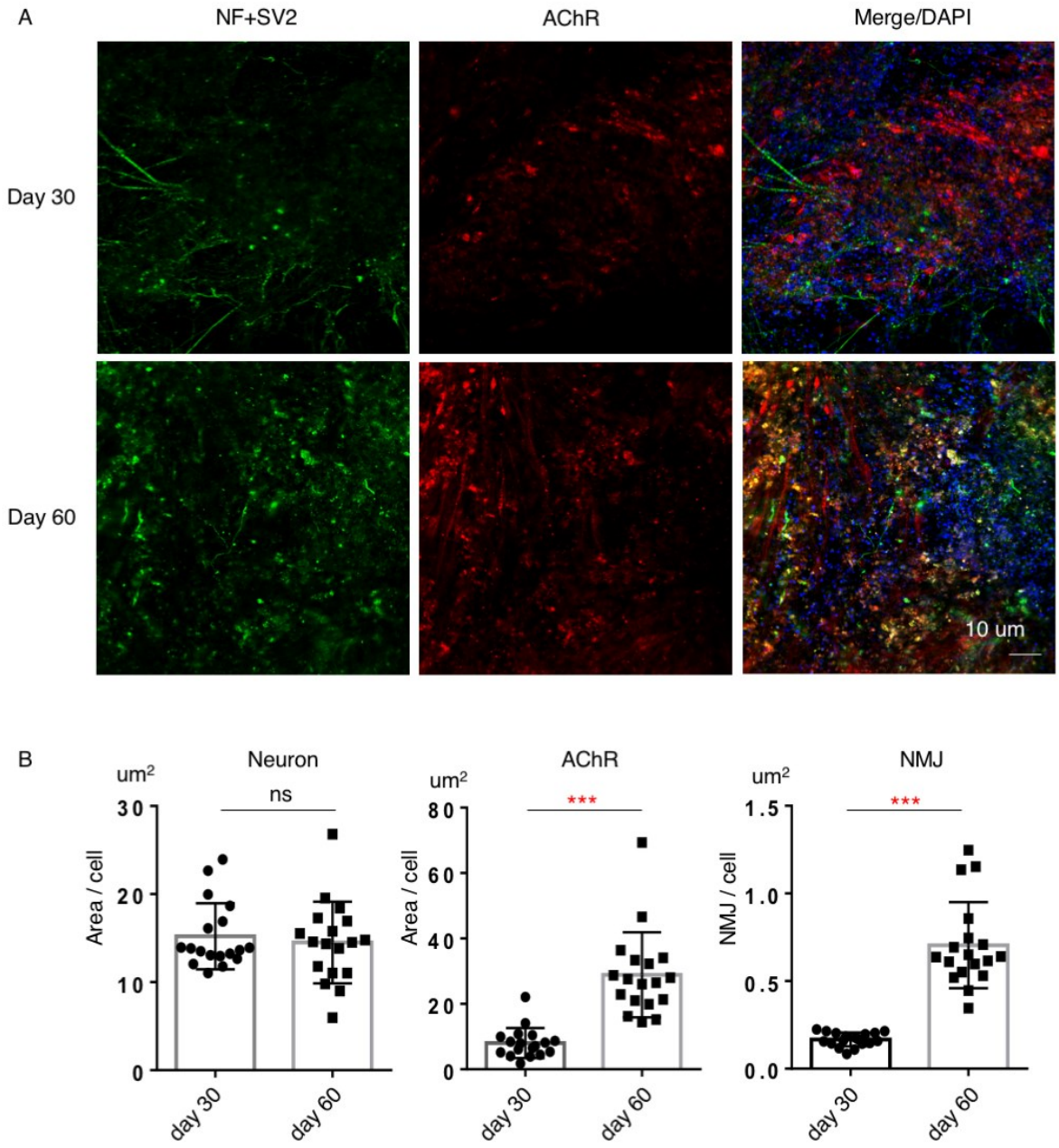
Supplemental Figure 4. Cell-to-cell contacts are required for myogenic differentiation. (A) Scheme of the experiment. 201B7^{GFP} were co-cultured with differentiating myotubes or cultured in conditioned culture (conditioned medium for NMJ differentiation), neuronal culture or myogenic culture. **(B)** Representative images of MHC+ myotubes and Tuj1+ neurons differentiated from 201B7^{GFP} co-cultured with differentiating myotubes on day 20. MHC+/GFP+ cells are shown by blue arrowheads. **(C)** Representative images for each experimental condition. **(D)** Quantitative immunocytochemistry analysis of the MHC+ or Tuj1+ area to GFP+ area. n=3. ****: $p<0.0001$, **: $p<0.01$, *: $p<0.05$ (one-way ANOVA with Dunnett's test relative to co-cultured condition).

Supplemental Figure 5



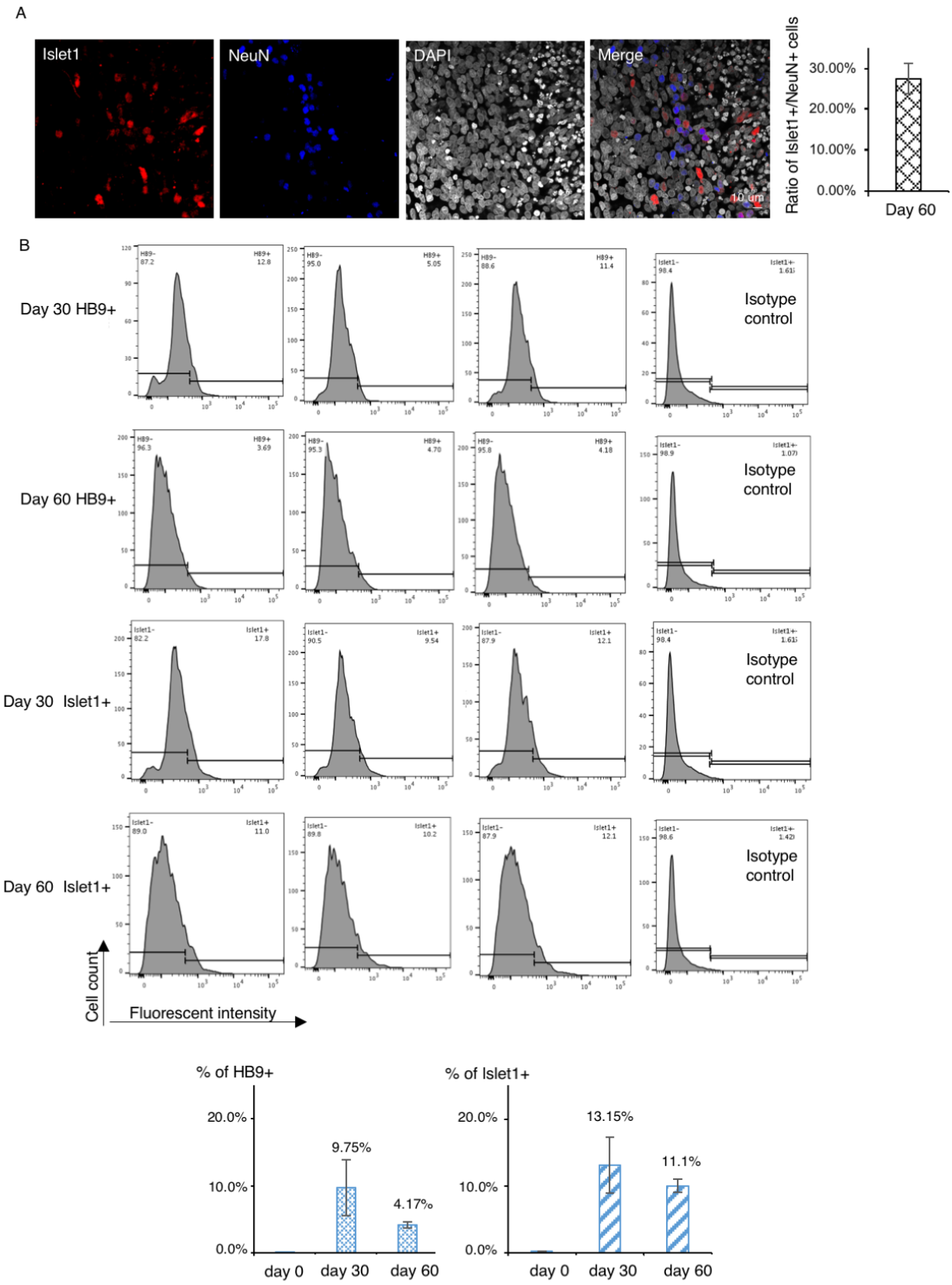
Supplemental Figure 5. Detection of AChR gamma and epsilon subunits. (A-C) The time-series gene expressions of AChR subunits gamma (A) and epsilon (B) in hNMJ culture. (C) The ratio of epsilon to gamma subunits. **(D)** Representative images and quantification results of AChR-gamma (AChR-G) and AChR-epsilon (AChR-E). Days indicate the time after Dox administration. ***: $p<0.001$ (unpaired Student's two-sided t-test). Data are means ± SEM. n=3 (A-C), n=12 (D). Each dot represents a biologically independent sample.

Supplemental Figure 6

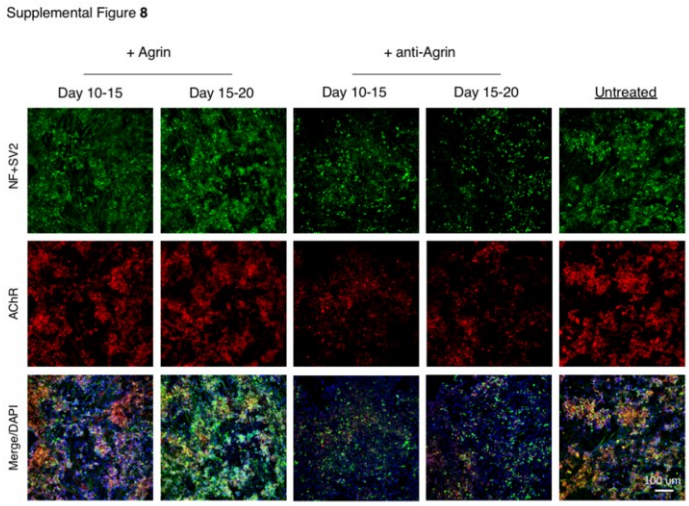


Supplemental Figure 6. Morphological changes in hNMJ culture. (A) Immunocytochemistry detection of neurons, AChRs (aBTX) and NMJs. **(B)** Quantitative analysis of the areas of neurons, AChRs and NMJs. ***: $p<0.001$ (unpaired Student's two-sided t-test). Data are means ± SEM. n=18. Each dot represents a biologically independent sample.

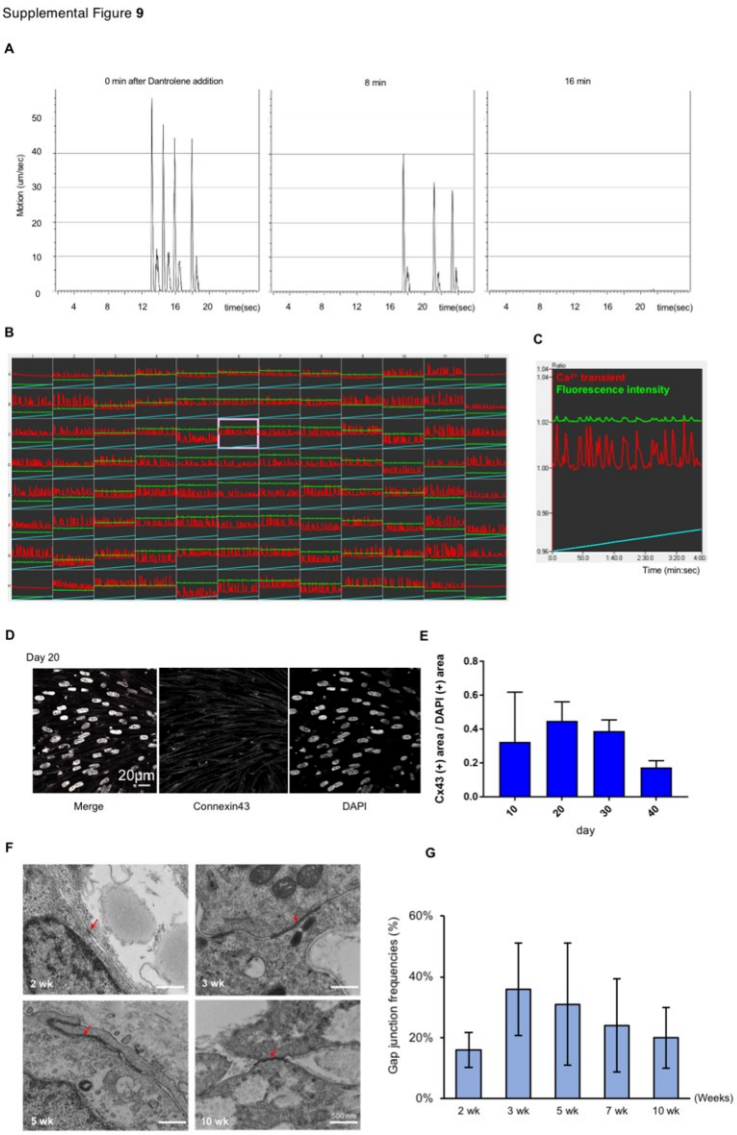
Supplemental Figure 7



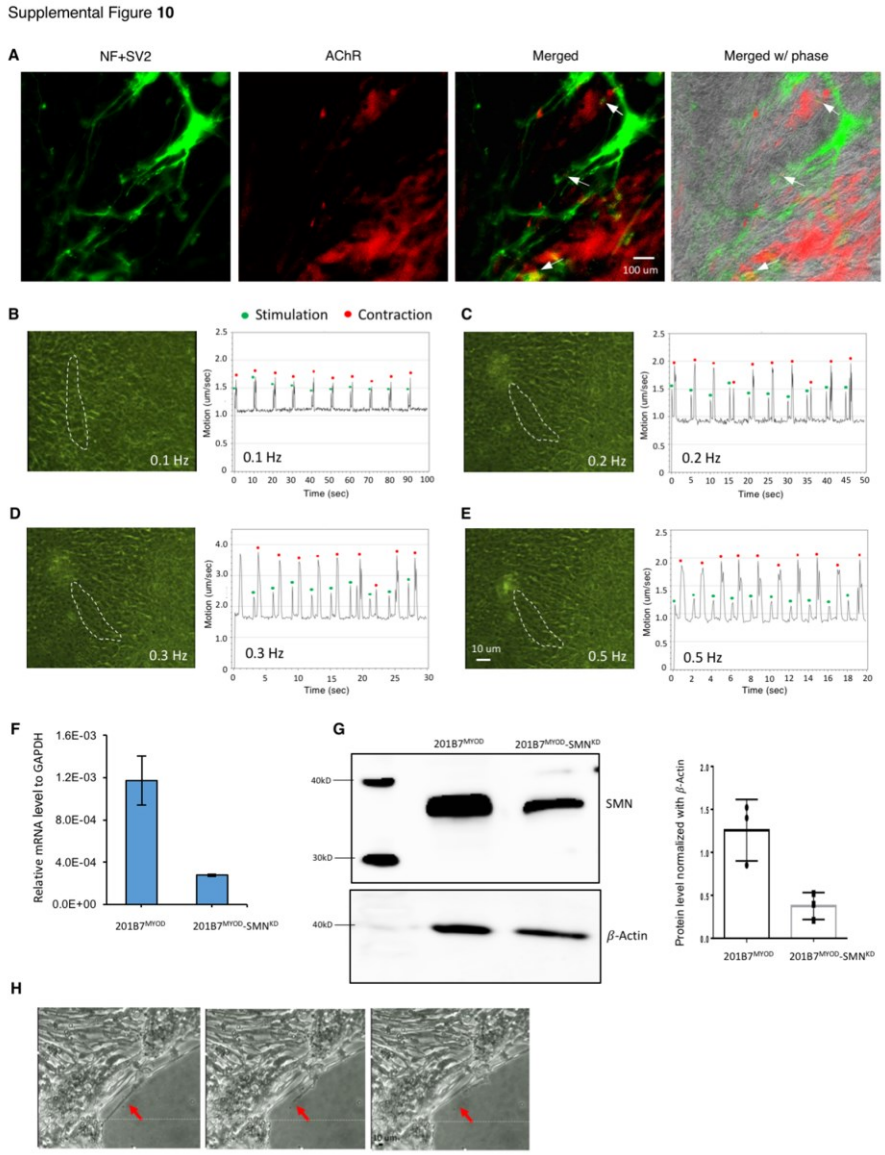
Supplemental Figure 7. MN detection and quantification in hNMJ culture. (A) Representative images of MN-specific markers detected at day 60. **(B)** The HB9+ and Islet1+ cells detected in total cells of NMJ culture at day 30 and day 60 by flow cytometry analysis. Data are means ± SEM. n=2 (A) and n=3 (B) biologically independent samples.



Supplemental Figure 8. Representative images of Agrin and anti-Agrin treatment. Related to Figure 3K.

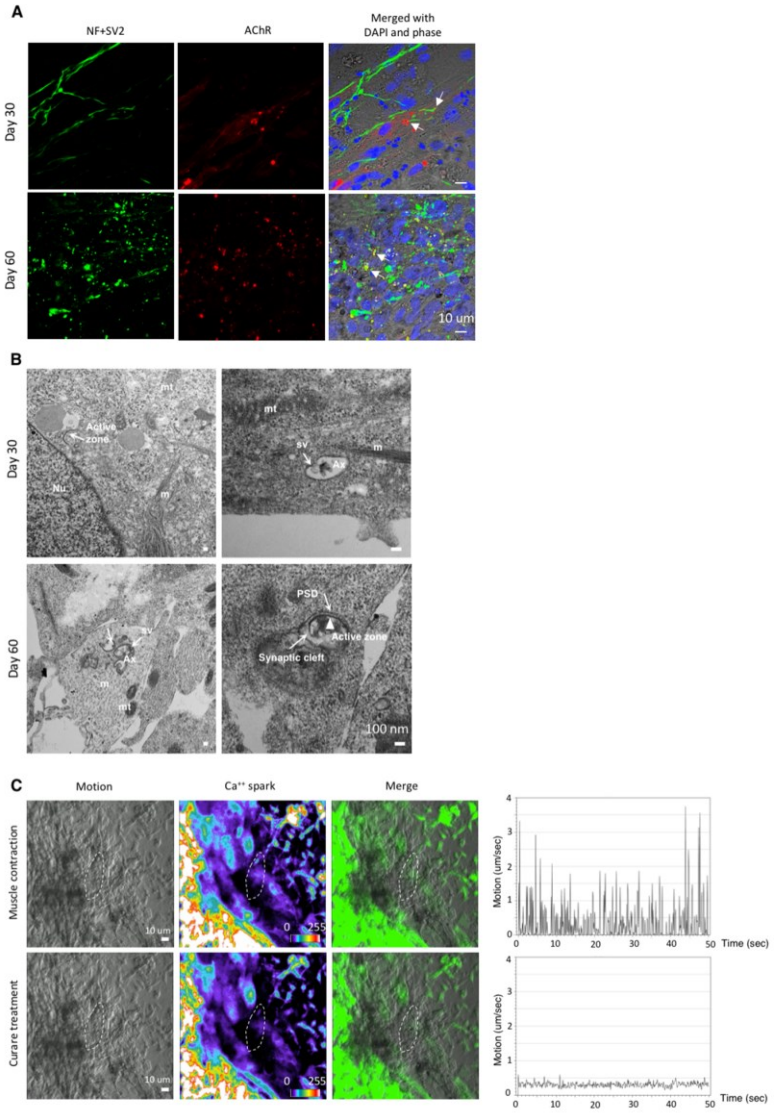


Supplemental Figure 9. Functional analysis at day 15-30 of NMJ differentiation (A-C) and gap junction detection (D-G), related to Figure 4. (A) Motion analysis of muscle contractions. Administration of the skeletal muscle relaxant Dantrolene inhibited muscle contraction. **(B)** Robust Ca^{2+} oscillations in myotubes were observed in all wells of a 96-well plate. Cells were not seeded in the four corners. **(C)** Magnification of the framed well in (B). (B, C) Green: fluorescence intensity, red: Ca^{2+} transients. **(D)** Representative images of connexin43 formed on the membrane of myotubes. **(E)** Quantitative immunocytochemistry analysis of the connexin43+ area to the DAPI+ area. **(F)** Gap junction morphology observed in TEM images (arrows) in myotubes at 2, 3, 5 and 10 weeks. Scale bars = 500 nm. **(G)** Quantitative analysis of the frequency of gap junctions in TEM images.



Supplemental Figure 10. Optogenetics experiments in NMJ culture and analysis of SMN gene knockdown iPSCs. (A) Immunocytochemistry staining for NMJ structures (arrowheads) in culture at day 100. **(B-E)** Synchronous muscle contractions triggered by pulsed blue light in NMJ cultures. Images and motion analysis of the optogenetics at 0.1, 0.2, 0.3 and 0.5 Hz pulsed blue light. See also Movies 5-8. **(F)** SMN gene expression of control and SMN knockdown iPSCs. **(G)** Western blotting and quantification of SMN protein expression. **(H)** Representative bright field images of 201B7^{MYOD}-SMN^{KD} myofibers breaking during contraction (red arrows). (F and G) Data are means ± SEM, n=3.

Supplemental Figure **11**



Supplemental Figure 11. NMJs differentiated from human ES cells (H9^{MYOD}) at day 30 and day 60. (A) Detection of neurons and AChRs in NMJ culture by immunocytochemistry staining. Arrows indicate NMJs. **(B)** TEM images of mature NMJ. Ax, axon terminal; m, myotube; mt, mitochondria; Nu, nucleus; PSD, postsynaptic density; sv, synaptic vesicle. **(C)** Muscle contraction and Ca^{++} imaging of NMJ culture at day 30. The dotted area indicates the area used for the motion analysis before and after Curare treatment. See also Movies 12 and 13.

Movie 1 for Figure 5, Supplementary_movie_1.mp4
Movie 2 for Figure 5, Supplementary_movie_2.mp4
Movie 3 for Figure 6, Supplementary_movie_3.mp4
Movie 4 for Figure 6, Supplementary_movie_4.mp4
Movie 5 for Figure 6, Supplementary_movie_5.mp4
Movie 6 for Figure 6, Supplementary_movie_6.mp4
Movie 7 for Figure 6, Supplementary_movie_7.mp4
Movie 8 for Figure 6, Supplementary_movie_8.mp4
Movie 9 for Figure 7, Supplementary_movie_9.mp4
Movie 10 for Figure 7, Supplementary_movie_10.mp4
Movie 11 for Figure 7, Supplementary_movie_11.mp4
Movie 12 for Supplemental Figure. 11, Supplementary_movie_12.mp4
Movie 13 for Supplemental Figure. 11, Supplementary_movie_13.mp4

Table S1 - Table S1.xlsx

Full unedited gel for Supplemental Figure 10