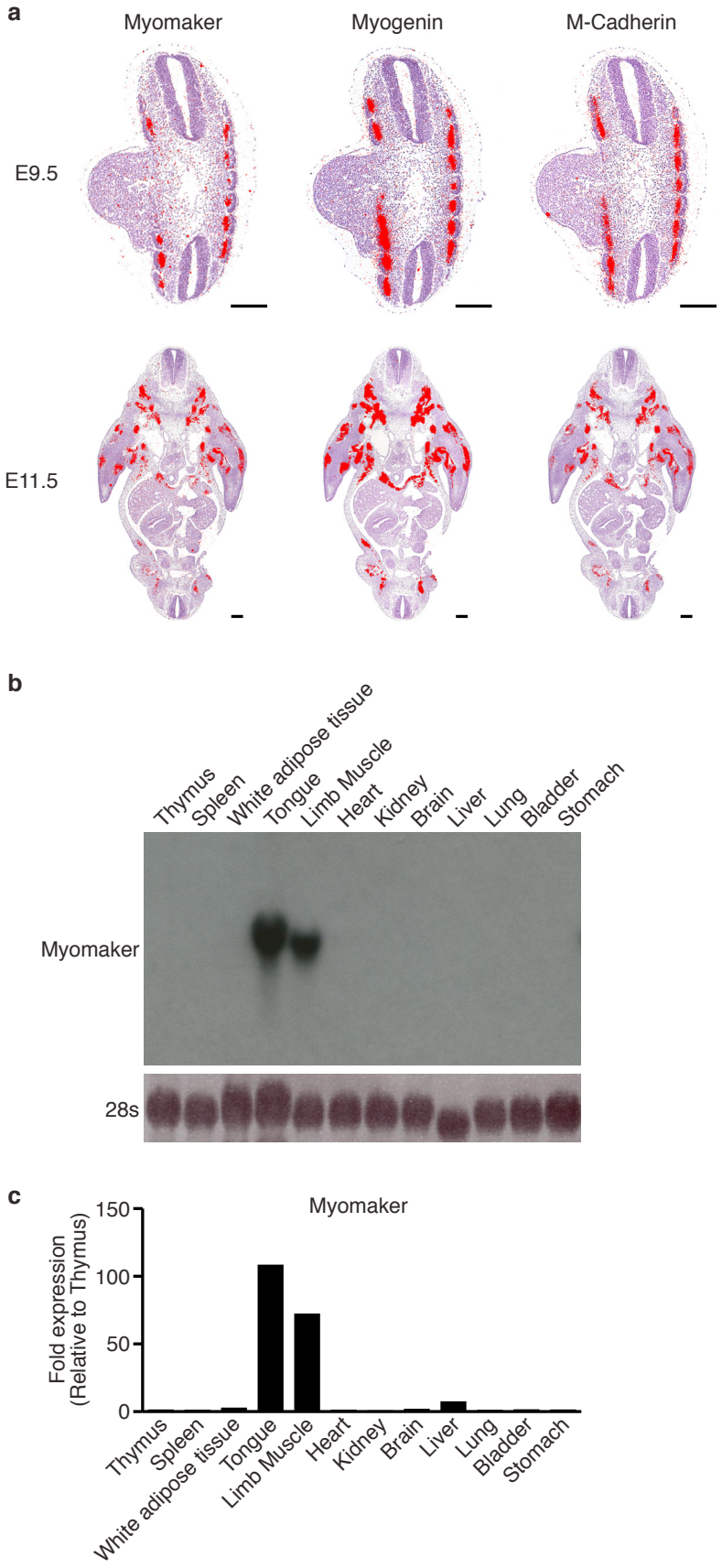
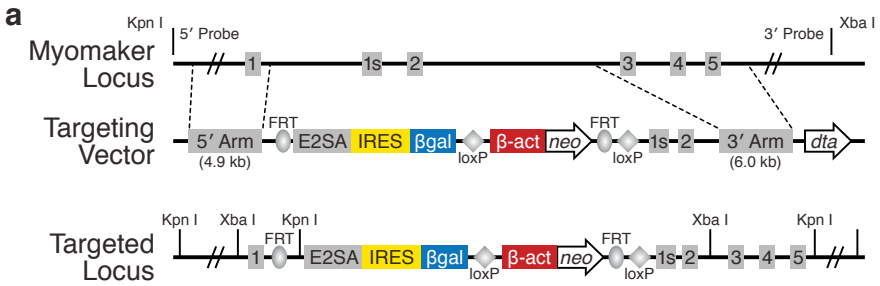
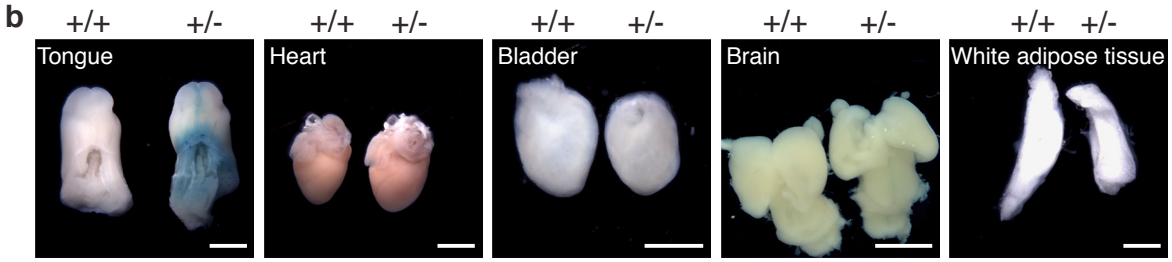
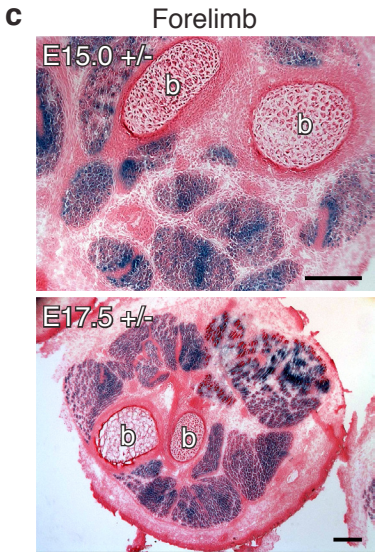
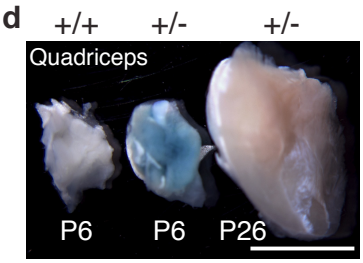


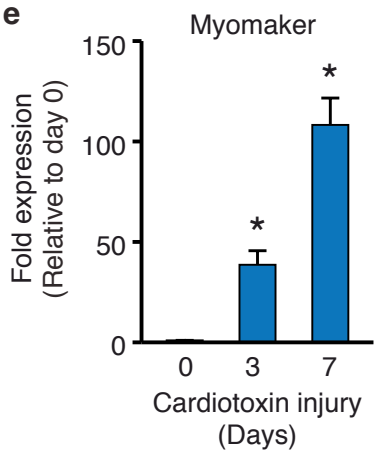
Supplementary Figure 1.
***Myomaker* is skeletal muscle-specific in the embryo.** **a,** E9.5 and 11.5 embryos were sectioned transversely and radioisotopic in situ hybridizations for *Myomaker*, *Myogenin*, and *M-cadherin* were performed. Each transcript exhibited expression in the somites at E9.5 and in the entire myotome of E11.5 embryos. *Myomaker* is expressed at relatively lower levels at E9.5 compared to E11.5. The top of the embryos pictured is rostral and the bottom caudal. Images were captured using darkfield microscopy, converted to red pseudo color, and overlayed on a brightfield image. **b,** RNA was isolated from multiple tissues of E19 wild-type embryos and assessed for the presence of Myomaker transcripts by Northern blot. **c,** Quantitative real-time PCR for Myomaker on the same tissues as in (**b**). Scale bars: 200 μm.

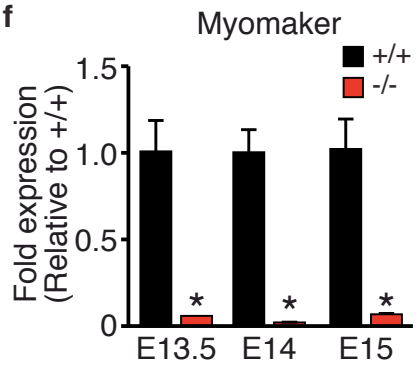








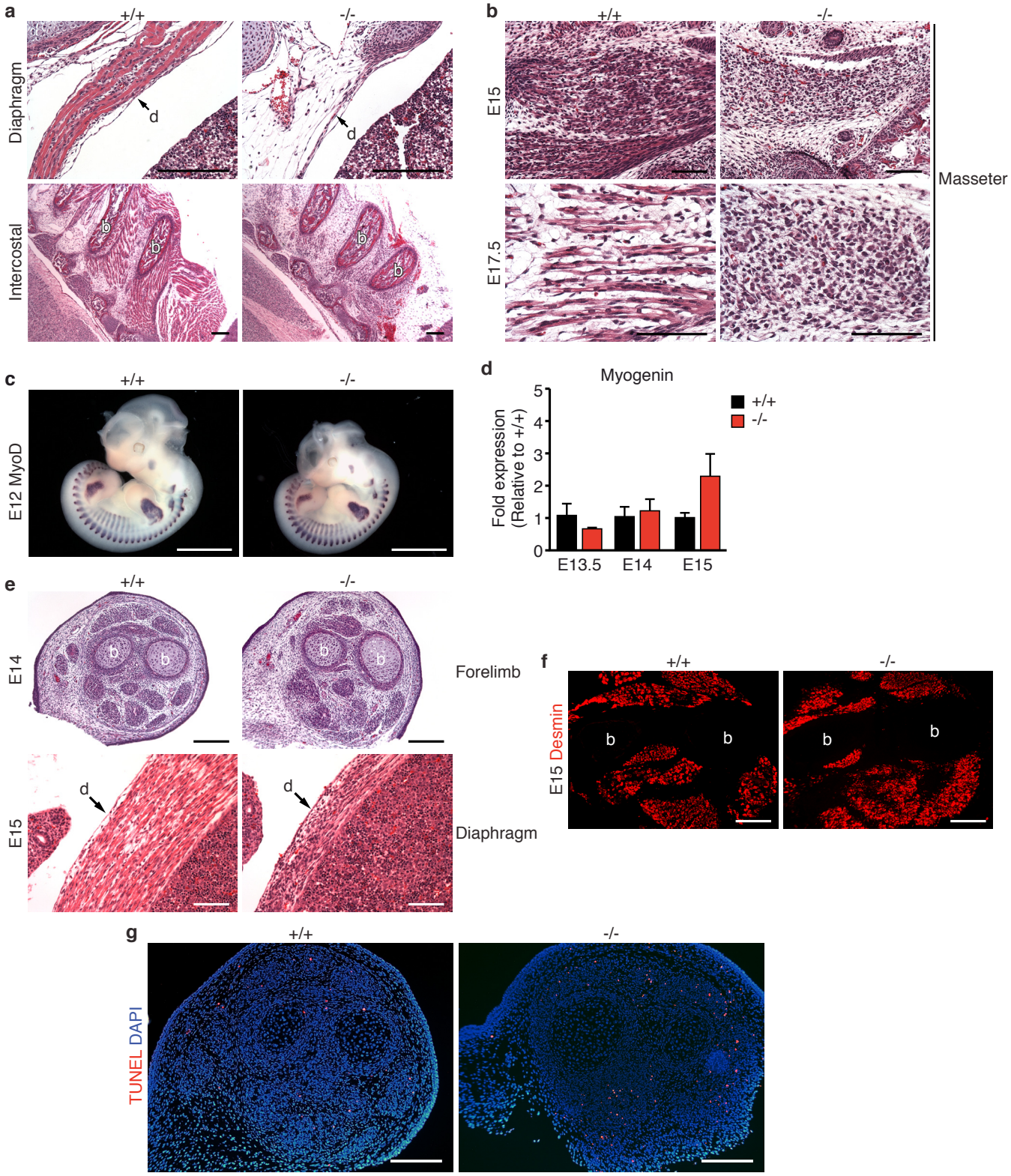




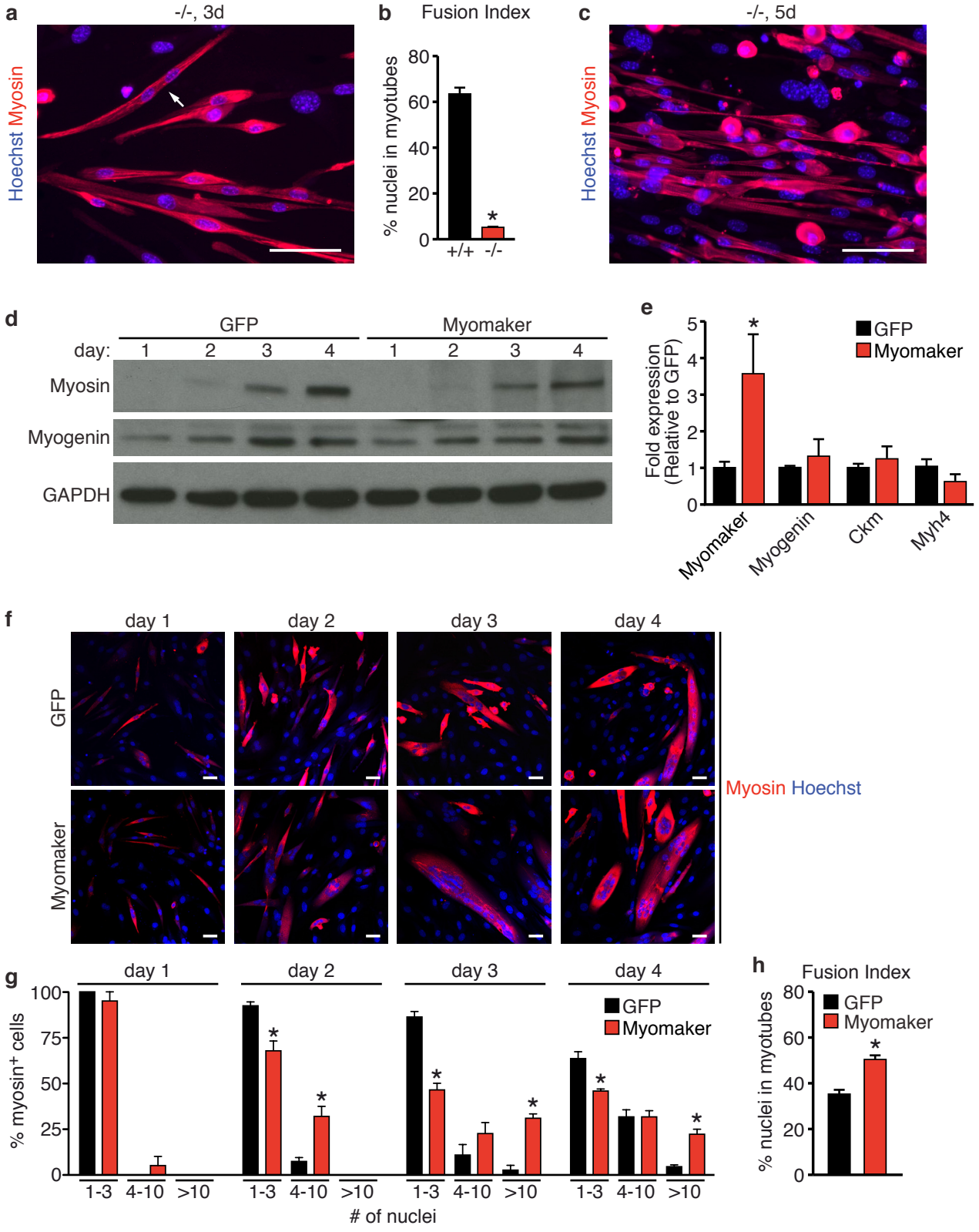
g

			myomaker		
Age	Total pups		+/+	+/-	-/-
E15	50	Observed	13 (26%)	24 (48%)	13 (26%)
		Expected	13 (25%)	25 (50%)	13 (25%)
E17.5	25	Observed	8 (32%)	11 (44%)	6 (24%)
		Expected	6 (25%)	13 (50%)	6 (25%)
P7	57	Observed	24 (42%)	33 (58%)	0
		Expected	14 (25%)	29 (50%)	14 (25%)

Supplementary Figure 2.
The Myomaker-LacZ allele recapitulates expression of Myomaker RNA and loss of Myomaker is lethal. **a,** An IRES-β-galactosidase and neomycin cassette was inserted in intron 1. The strong splice acceptor site (E2SA) results in a *Myomaker-LacZ* fusion and interruption of downstream transcription, generating a null allele. These targeted ES cells were obtained from KOMP as described in Materials and Methods. **b,** Tissues harvested from P6 *Myomaker*$^{+/-}$ mice were stained with X-gal. *Myomaker-LacZ* expression was not detected in any non-skeletal muscle tissues. **c,** X-gal staining on forelimbs from *Myomaker*$^{+/-}$ embryos demonstrates expression in muscle fibers. The b denotes bones. **d,** Quadriceps from P6 WT, and P6 and P26 Myomaker+/- mice were stained with X-gal to visualize postnatal down-regulation of the Myomaker-LacZ allele. **e,** WT tibialis anterior (TA) muscle was injured with cardiotoxin and analyzed for Myomaker transcripts by qPCR at day 0, 3, and 7 after injury. **f,** Myomaker RNA was absent from the tongue muscle of E13.5, E14, and E15 *Myomaker*$^{-/-}$ embryos. Data are presented as mean ± SEM. * $P < 0.05$ compared to +/+. **g,** Myomaker null embryos were obtained at expected Mendelian frequencies at E15 and E17.5. However, we did not observe a *Myomaker* knockout at P7 due to lethality. Scale bars: **b**, **d**, 2 mm; **c**, 200 µm.



Supplementary Figure 3.
Myomaker is necessary for proper muscle formation despite normal specification. a, Paraffin sectioning and H&E staining on diaphragm and intercostal muscles reveal a lack of muscle fibers in E17.5 *Myomaker*$^{-/-}$ embryos. The b depicts bones. The d denotes diaphragm. **b,** Masseter muscle from E15 and E17.5 WT and *Myomaker*$^{-/-}$ embryos was paraffin sectioned and H&E stained to demonstrate the necessity of Myomaker for formation of skeletal muscles of the head. **c,** MyoD in situ hybridization shows comparable expression in E12 WT and *Myomaker*$^{-/-}$ embryos. **d,** qPCR for myogenin revealed no differences at the indicated ages in the tongues of WT (n=3) and Myomaker (n=3) null mice. Data are presented as mean ± SEM. **e,** E14 forelimbs and E15 diaphragm were sectioned and stained with routine H&E. *Myomaker*$^{-/-}$ muscle is present at this stage of development in the forelimbs and diaphragm. The b denotes bones of the limb. The d shows the diaphragm. Scale bars, 200 µm for forelimb and 100 µm for diaphragm. **f,** Desmin staining on WT and *Myomaker*$^{-/-}$ E15 forelimbs demonstrates the presence of muscle cells. The b denotes bones. **g,** E15 forelimbs were stained for DNA fragmentation with the TUNEL reaction and co-stained with DAPI. Apoptotic nuclei were more readily detected in *Myomaker*$^{-/-}$ muscle compared to WT muscle. Scale bars: **a**, **b**, **f**, **g** 200 µm; **c**, 2 mm



Supplementary Figure 4.

Myomaker governs fusion and not the levels of myogenic proteins. a, Myomaker null myoblasts were differentiated and we observed a small number of bi-nucleated cells (arrow). **b,** The fusion index was calculated as the percentage of nuclei contained in myotubes (a myosin$^+$ cell with at least two nuclei). **c,** *Myomaker*$^{-/-}$ myoblasts failed to fuse even after 5 days of differentiation. **d,** Western blots for myosin and myogenin were performed on C2C12 cells on the indicated day of differentiation after GFP or Myomaker infection. **e,** qPCR for myogenic differentiation genes revealed that Myomaker-mediated fusion enhancement did not alter levels of differentation (n=3 for GFP and n=3 for Myomaker). **f,** C2C12 myoblasts were immunostained with myosin and Hoechst (nuclei) to evaluate the kinetics of fusion after GFP or Myomaker infection. Myomaker-infected C2C12s exhibited an increase rate of appearance of myotubes with multiple nuclei. On day 3, myotubes with >20 nuclei were apparent in C2C12 cells with over-expressed Myomaker, whereas these myotubes were not detected in control cultures on this day. **g,** Quantitation of the percentage of myosin$^+$ cells that contained the indicated number of nuclei on day 1 – day 4 of differentiation. Data are presented as mean ± SEM. * $P < 0.05$ compared to GFP-infected cells on that day of differentiation. **h,** The fusion index on day 4 of differentiation was quantified as in (**b**) and showed an increase in myotube nuclei after Myomaker infection.

Scale bars: **a**, **c**, 100 µm, **f**, 20 µm.

a

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Human       MGTLVAKLLLPTLSSLAFLPTVSIAAKRRFHMEAMVYLFTLFFVALHHACNGPGLSVLCF 60
Dog         MGTLAAKLLLPTLSSLAFLPTVSIAAKRRFHMEAMVYLFTMFFVALHHACNGPGLSVLCF 60
Pig         MGTVMAKLLLPTLSSLAFLPTVSIAAKRRFHMEAMVYLFTTFFVAFYHACHGPGLAMICF 60
Mouse       MGTVVAKLLLPTLSSLAFLPTVSIATKRRFYMEAMVYLFTMFFVAFSHACDGPGLSVLCF 60
Oppossum    MGTLVTKLLLPTISSLAFLPTISIAAKRRFHMEAMVYLFTMFFIAIYHACDGPGLSVLCF 60
Zebrafish   MGAFIAKMLLPTISSLVFVPAASVAAKRGFHMEAMVYFFTMFFTAIYHACDGPGLSILCF 60
            **:. :*:****:***.*:*: *:*:** *:*******:** ** *: ***.****:::**

Human       MRHDILEYFSVYGTALSMWVSLMALADFDEPKRSTFVMFGVLTIAVRIYHDRWGYGVYSG 120
Dog         MRHDVLEYFSVYGTALSMWVSLMALADFDEPKRSTFVMFGVLTIAVRIYHDRWGYGVYSG 120
Pig         LRLDILEYFSVYGTALSMWVSLMALADFDEPKRSTFVMFGVLTIAVRIYHDRWGYGVYSG 120
Mouse       MRRDILEYFSIYGTALSMWVSLMALADFDEPQRSTFTMLGVLTIAVRTFHDRWGYGVYSG 120
Oppossum    MRYDILEYFSIYGTALSMWVSLMALAEFDEPKRSTFVMFGVLTIAVRIYQDRWGYGVYSG 120
Zebrafish   MKYDILEYFSVYGTAISMWVTLLALGDFDEPKRSSLTMFGVLTAAVRIYQDRLGYGIYSG 120
            :: *:*****:****:****:*:**.:****:**::.*:**** *** ::** ***:***

Human       PIGTAILIIAAKWLQKMKEKKGLYPDKSVYTQQIGPGLCFGALALMLRFFFEDWDYTYVH 180
Dog         PIGTAVLIIATKWLQQMKEKKSLYPDKSVYTQQIGPGLCFGALALMLRFFFEDWDYTYVH 180
Pig         PIGTAALIIAAKWLQQMKDQRRLYPDKSVYTQQIGPGLCFGALALMLRFFFEEWDYTYVH 180
Mouse       PIGTATLIIAVKWLKKMKEKKGLYPDKSIYTQQIGPGLCFGALALMLRFFFEEWDYTYVH 180
Oppossum    PIGTAVLIIATKWLQKMKEKKGLYPDKSVYTQQIGPGFCFGALALMLRFFFQEWDYTYVH 180
Zebrafish   PIGTAVFMITVKWLQKMKEKKGLYPDKSVYTQQVGPGCCFGALALMLRFYFEEWDYAYVH 180
            ***** ::*:.***::**::: ******:****:*** ***********:*::***:***

Human       SFYHCALAMSFVLLLPKVNKKAGSPGTPAKLDCSTLCCACV 221
Dog         SFYHCALAMSFVLLLPKVNKKAGSAGPPAKLDCSTLCCACI 221
Pig         SFYHCALAMSFVLLLPKANKKAGSAGPPAKLDCSTLCCACI 221
Mouse       SFYHCALAMSFVLLLPKVNKKAGNAGAPAKLTFSTLCCTCV 221
Oppossum    SFYHCSLAMSFVLLLPKVNKKAGNAGTPAKLDCSTLCCACI 221
Zebrafish   SFYHVSLAMSFILLLPKKNRYAGTGRNAAKLNCYTLCCCV- 220
            **** :*****:***** *: **.   .***   ****
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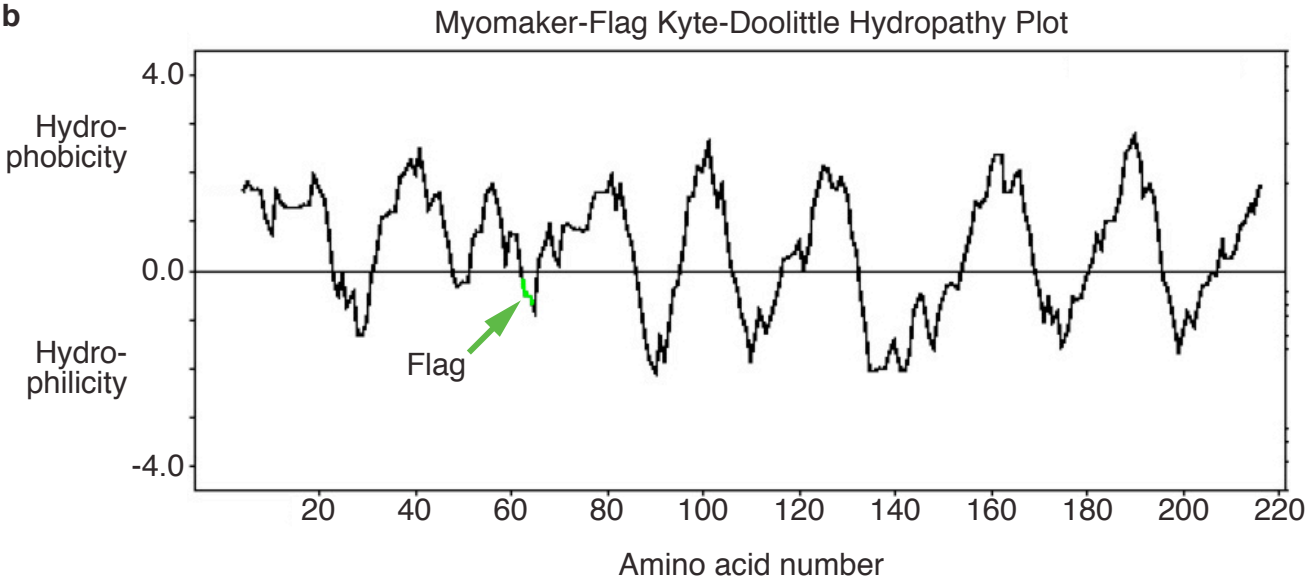
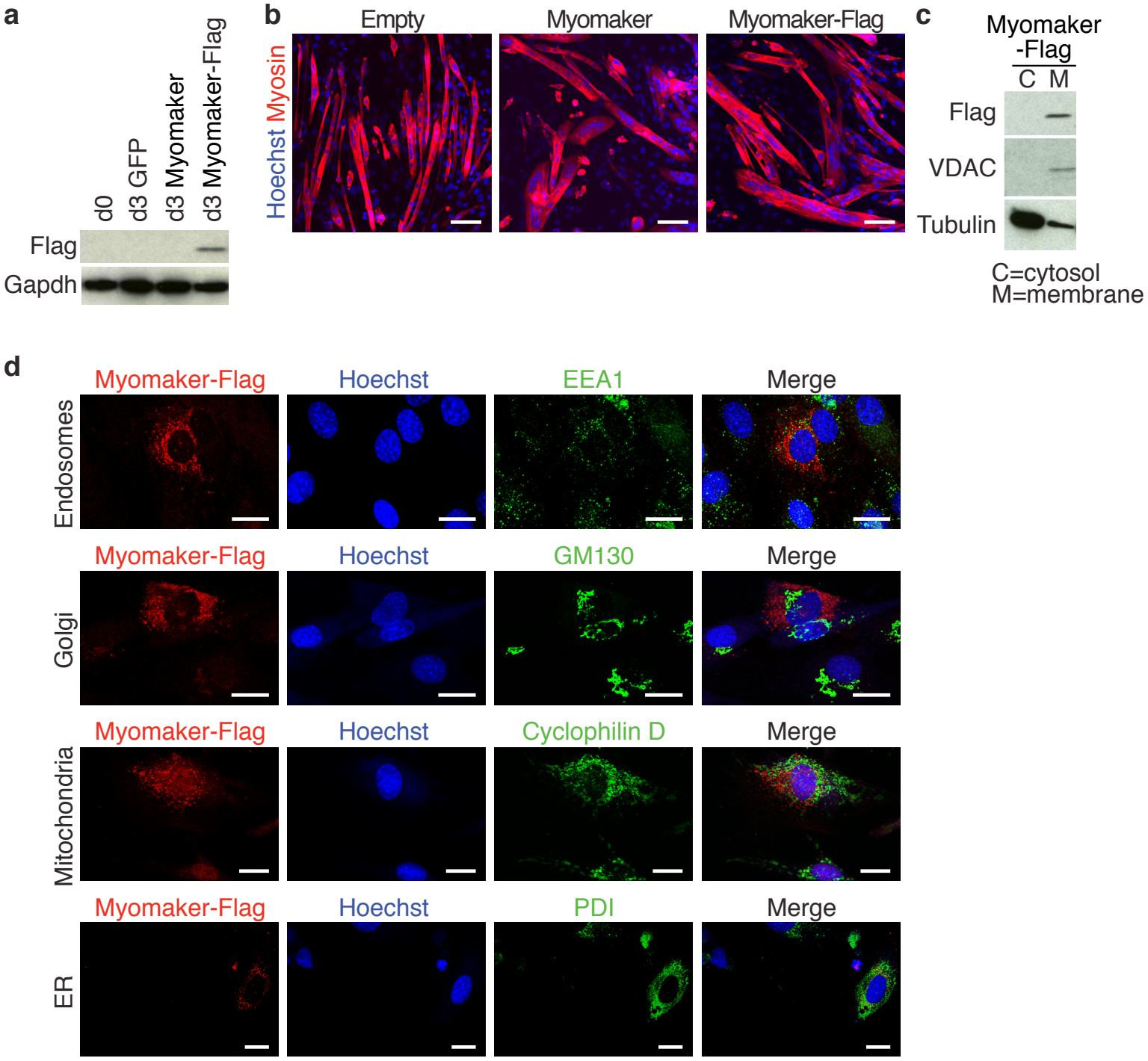
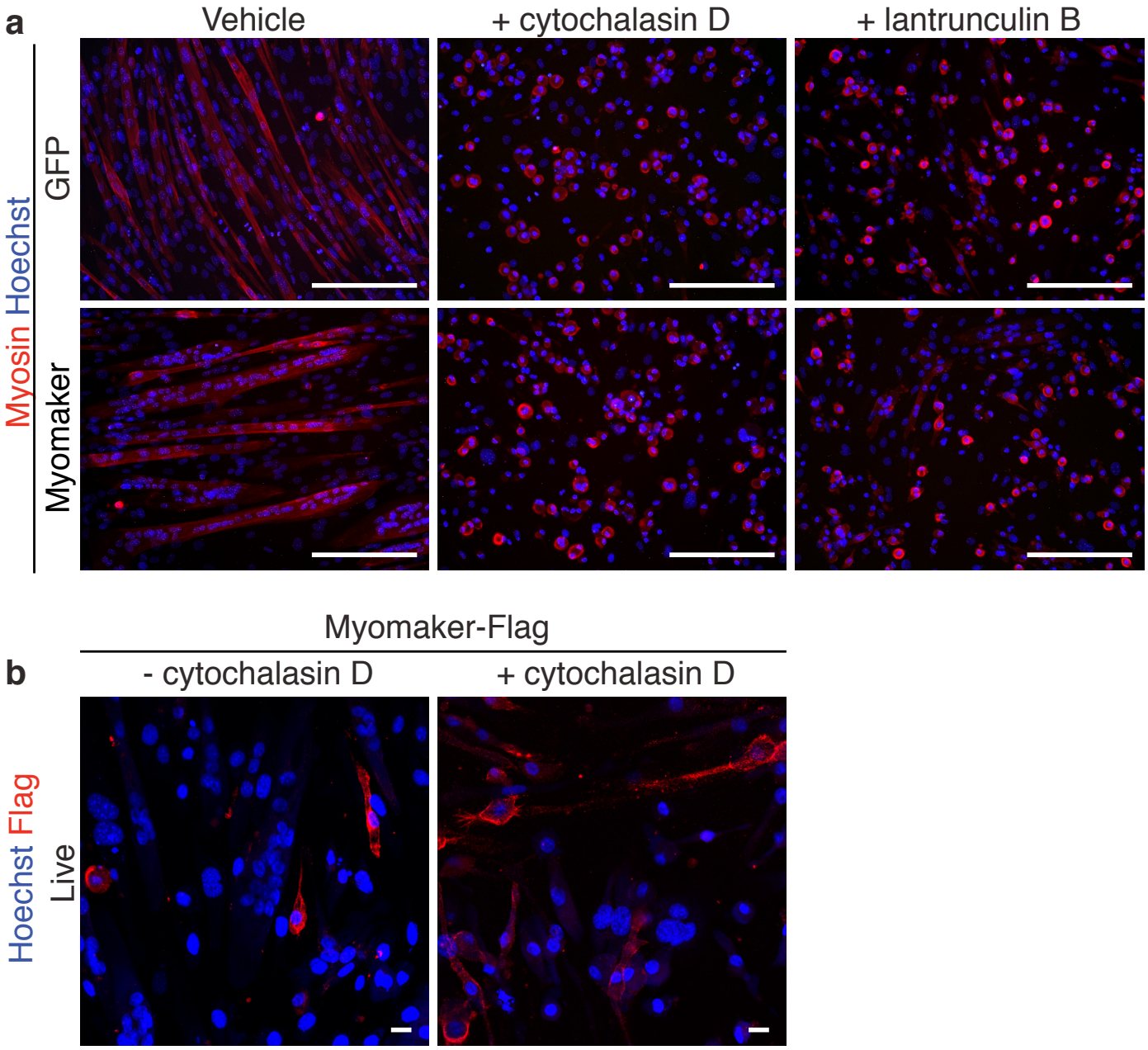
b



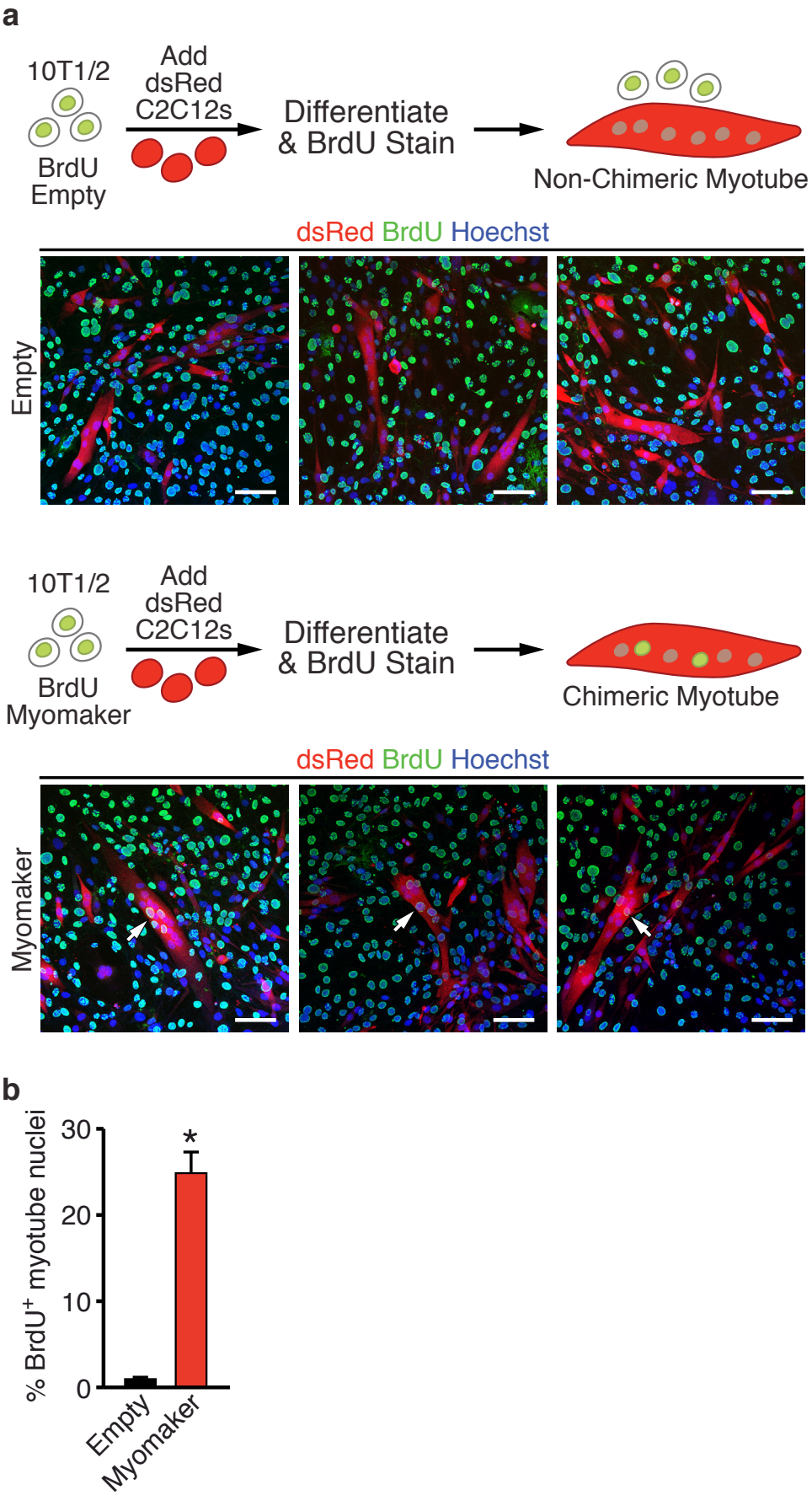
Supplementary Figure 5.
Myomaker amino acid conservation and hydrophobicity. a, Amino acid alignment of Myomaker proteins from Human, Dog, Pig Mouse, Oppossum, and Zebrafish shows strong conservation. **b,** Kyte-Doolittle plot for Myomaker-Flag shows its highly hydrophobic nature. Regions above the horizontal line are considered hydrophobic. Flag, denoted by the green line, was engineered after amino acid 61 of Myomaker.



Supplementary Figure 6.
Myomaker-Flag localizes to membrane compartments. **a,** C2C12 cells were infected with the indicated retroviruses and a Western blot analysis using a Flag antibody detected expression of Myomaker-Flag in whole cell lysates. **b,** Myomaker-Flag was over-expressed in C2C12 cells to test for its ability to increase myoblast fusion at levels similar to untagged Myomaker. Cells were stained 4 days after differentiation. **c,** C2C12 cells were lysed in hypotonic buffer and the cytosol (C) and membrane (M) fractions were obtained using differential centrifugation. Western blot analysis using a Flag antibody detected Myomaker-Flag protein only in the membrane fraction. Detection of VDAC for the membrane fraction and Tubulin for the cytosolic fraction was used to show efficient separation of the cellular compartments. **d,** C2C12 cells were infected with Myomaker-Flag and immunostained on day 2 of differentiation to assess co-localization with known proteins that are specific for certain intracellular compartments. Specifically, the cells were fixed and permeabilized and stained with Flag antibody and either Early Endosome Antigen 1 (EEA1, endosomes), Golgi matrix protein (GM130), Cyclophilin D (Mitochondria), or protein disulfide isomerase (PDI, endoplasmic reticulum). Nuclei were stained using Hoechst. Myomaker-Flag exhibited partial co-localization with endosomes and ER. Scale bars: 20 μm.



Supplementary Figure 7.
A functional actin-cytoskeleton is necessary for Myomaker function. **a,** C2C12 myoblasts were infected with either GFP- or Myomaker-retrovirus and differentiated in the presence of vehicle (0.1% EtOH), cytochalasin D, or lantrunculin. Myomaker over-expression did not overcome the deleterious effects of actin inhibition on myoblast fusion. **b,** Live staining of C2C12 cells after infection with Myomaker-Flag virus with and without cytochalasin D treatment demonstrates that actin dynamics does not regulate location of Myomaker on the cell surface. Scale bars: **a**, 200 µm; **b**, 20 µm.



Supplementary Figure 8.
BrdU⁺Myomaker⁺ fibroblasts fuse to C2C12 myoblasts. **a,** 10T1/2 fibroblasts were treated with BrdU overnight and then infected with either Empty- or Myomaker-retrovirus, followed by mixing with dsRed-infected C2C12 cells and induced to differentiate for 4 days. Analysis of BrdU incorporation in dsRed-myotubes (arrows) revealed Myomaker was sufficient to fuse fibroblasts to C2C12 cells. We observed empty-infected BrdU⁺ fibroblasts juxtaposed to dsRed-myotubes, but negligible fusion. **b,** Quantitation of the percentage of myotube nuclei that were BrdU⁺ in the indicated cultures. Scale bars: 100 μm.