Small-Molecule Fluorophores To Detect Cell-State Switching in the Context of High-Throughput Screening

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Supporting Information

Small-molecule fluorophores to detect cell-state switching in the context of high-throughput screening

Bridget K. Wagner*, Hyman A. Carrinski, Young-Hoon Ahn, Yun-Kyung Kim,
Tamara J. Gilbert, Dina A. Fomina, Stuart L. Schreiber,
Young-Tae Chang, and Paul A. Clemons*

Chemical Biology Program, Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, Massachusetts 02142

* Corresponding author email: bwagner@broad.harvard.edu; pclemons@broad.harvard.edu

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Supporting information

1. Methods

Cell culture

C2C12 myoblasts (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100μg/m*L* penicillin/streptomycin mixture) in a humidified atmosphere at 37 °C with 5% CO_2. Differentiation into myotubes was induced at 80% density on "day 0" by changing media to DMEM supplemented with 2% horse serum (DM). 3T3-L1 preadipocytes were grown in DMEM supplemented with 10% donor calf serum and antibiotics. Differentiation into adipocytes was induced two days after confluence by treating for 48 h with 175 nM insulin, 5 μM dexamethasone, and 500 μM 3-isobutylmethylxanthine in DMEM+FBS. Two days later, media was changed to DMEM+FBS containing insulin, and changed every two days thereafter; differentiation was complete (>90% adipocytes) after 10-14 days.

High-throughput and high-content screening for cellular fluorescence

For all screening experiments, 4000 C2C12 myoblasts were seeded per well of black 384-well optical bottom plates, at 50 μ*L*/well. On day 4 of differentiation, 100 n*L* compound was pin-transferred in duplicate into fresh media with a steel pin array, using the CyBi-Well robot (CyBio, Woburn, MA). In order to increase the number of mock-treated wells included in the control distribution, an additional plate was added, which received DMSO alone by pin-transfer into each well. Mock- and compound-treated

plates were incubated at 37 °C for 1-4 h (time of incubation did not result in any differences in outcome), followed by cell fixation, staining of DNA with Hoechst 33342, and washing with PBS. All high-throughput cell-based assay measurements were performed using the EnVision plate reader (PerkinElmer, Waltham, MA). For high-throughput screening, ChemBank "Composite-*Z*" scores, reflecting compound performance as compared to a mock-treated (DMSO) distribution, were calculated as described (Franz, 2007; Seiler, 2008).

High-content screening involved treating the plates as described above, with the exception of the measuring fluorescence with the ImageXpress IX5000A automated microscope (Molecular Devices; Sunnyvale, CA). Images from each well were acquired at 4X objective magnification at two wavelength pairs (485/530 nm, to detect compound fluorescence, and 360/460 nm, to detect nuclei). Exposure times were kept constant (DAPI channel 50 ms, FITC channel 25ms) in order to prevent saturation of the high-intensity images, and the resulting increased weight accorded dim features. Gamma correction, with an exponent of 0.6, was applied to all DAPI images in MATLAB (The Mathworks; Natick, MA), primarily to linearize the relationship between color and signal. Linear transformations of brightness and contrast were applied in exactly the same way to all FITC images in Photoshop (Adobe Systems Inc.; San Jose, CA).

High-throughput screen for myogenesis

For myogenesis screening experiments, C2C12 myoblasts were cultured and seeded as described above. The following day, 100-n*L* stock-solutions of various commercially available kinase inhibitors were pin-transferred into the assay plates in the

presence of DM. Cells were incubated with compounds for 48 h, then replaced with fresh compound-free DM. Cells were incubated for an additional 72 h, treated for 1 h with 1 μ*M* **E26** (see text), then fixed and washed as described above. Fluorescence was measured using the EnVision plate reader at 485/535 nm ($\lambda_{ex}/\lambda_{em}$).

2. Screening for cellular fluorescence

Quantitative data for high-throughput and high-content screening of the rosamine collection (Ahn, 2007) for fluorescence in myoblasts and myotubes is included in Tables S1, S2, and S3, respectively. Many features will cause a baseline fluorescence across an image. Since the values in this table are measured from raw intensity, the fluorescence is nonzero, as seen in columns “AllNucleiW1” and “AllNucleiW2”. The presence of zero in an intensity column (e.g., PositiveCellsW2) means that the green channel is without any object which meets the threshold based on the segmentation parameters, which are maintained for all images in the screen.

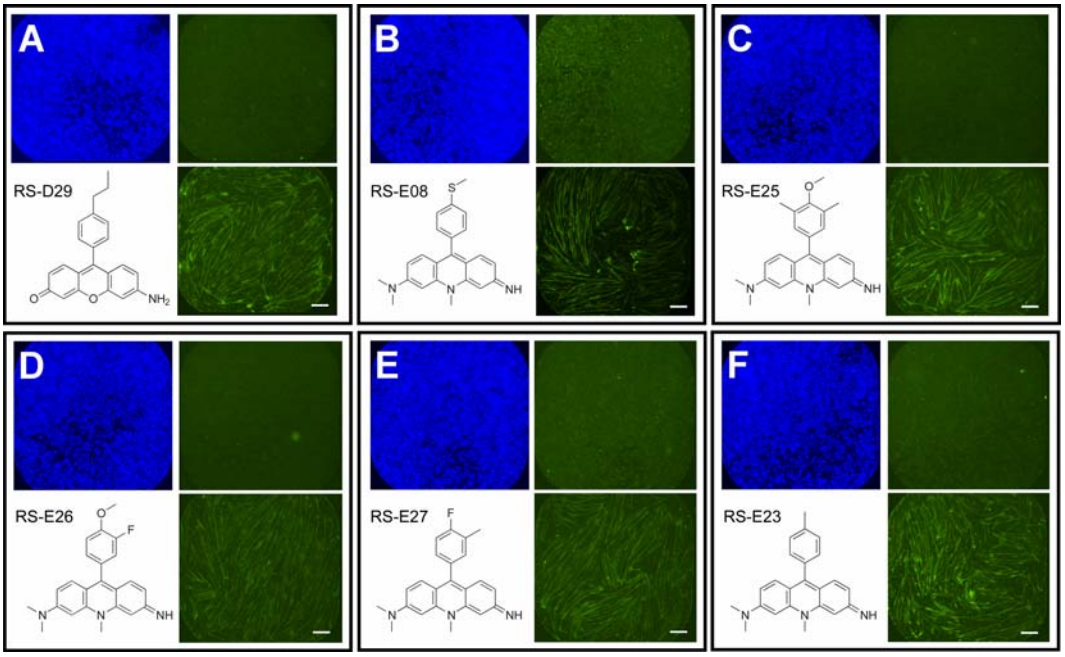

Figure S1. Representative images from high-content screening of rosamine (RS) library. Compounds were screened at 250 n*M* to detect cell type specificity: **(A)** compound **D29**, **(B)** compound **E08**, **(C)** compound **E25**, **(D)** compound **E26**, **(E)** compound **E27**, and **(F)** compound **E23**. For each panel, the images represent compound fluorescence in myoblasts (upper right), compound fluorescence in myotubes (lower right), identity and chemical structure of each compound (lower left), and nuclei of myoblasts (upper left), showing that wells of myoblasts and myotubes each contained comparable densities of nuclei. Scale bars = 150 μm.

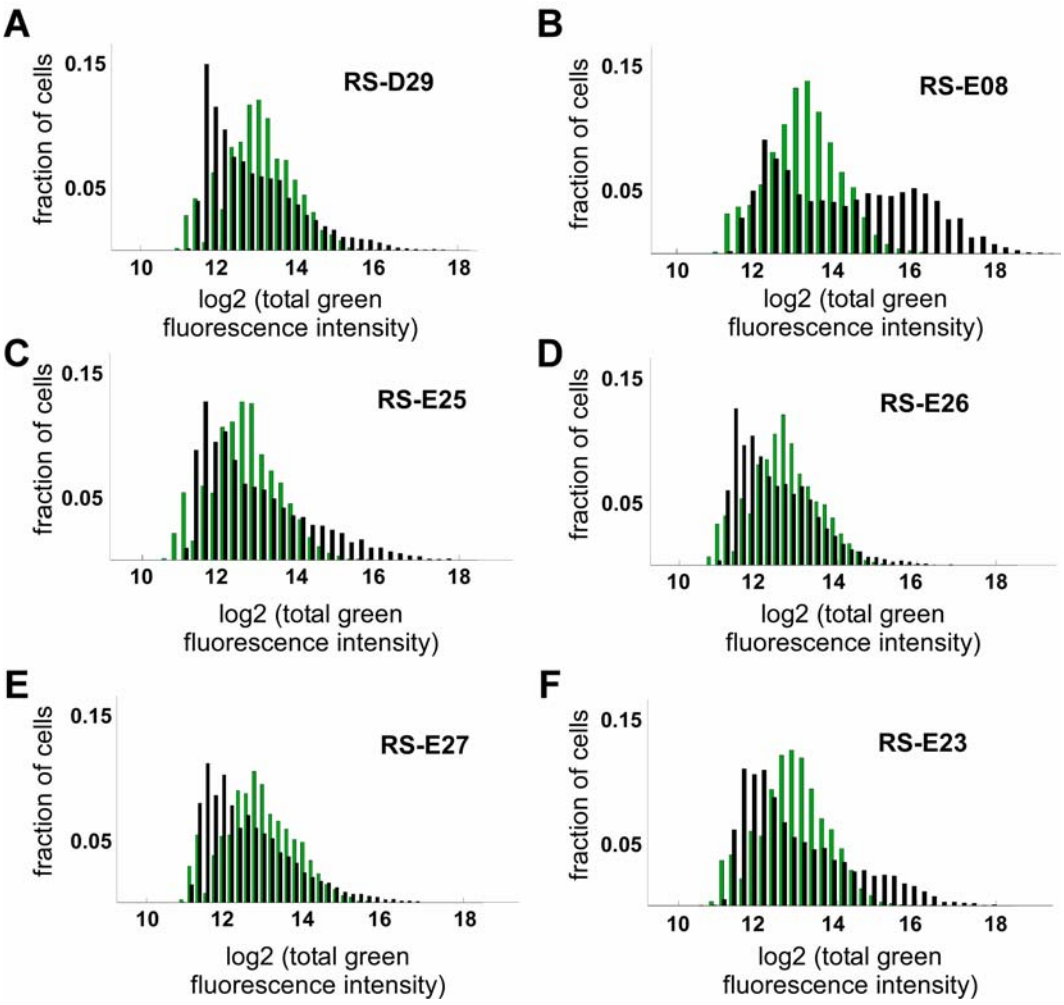

Figure S2. Histograms of fluorescence for each compound image represented in Figure S1: **(A)** compound **D29**, **(B)** compound **E08**, **(C)** compound **E25**, **(D)** compound **E26**, **(E)** compound **E27**, and **(F)** compound **E23**. Cells were segmented by the Cell Scoring module (Molecular Devices, Sunnyvale, CA) using the DAPI channel (Hoechst staining) for nuclei and the FITC channel for green fluorescence. Green fluorescence, expressed as logarithm-transformed total intensities, was quantified in the immediate vicinity of each nucleus. Black bars represent myoblasts treated with 250 n*M* of each compound, and green bars represent myotubes under the same conditions.

3. Compound retesting and cellular characterization

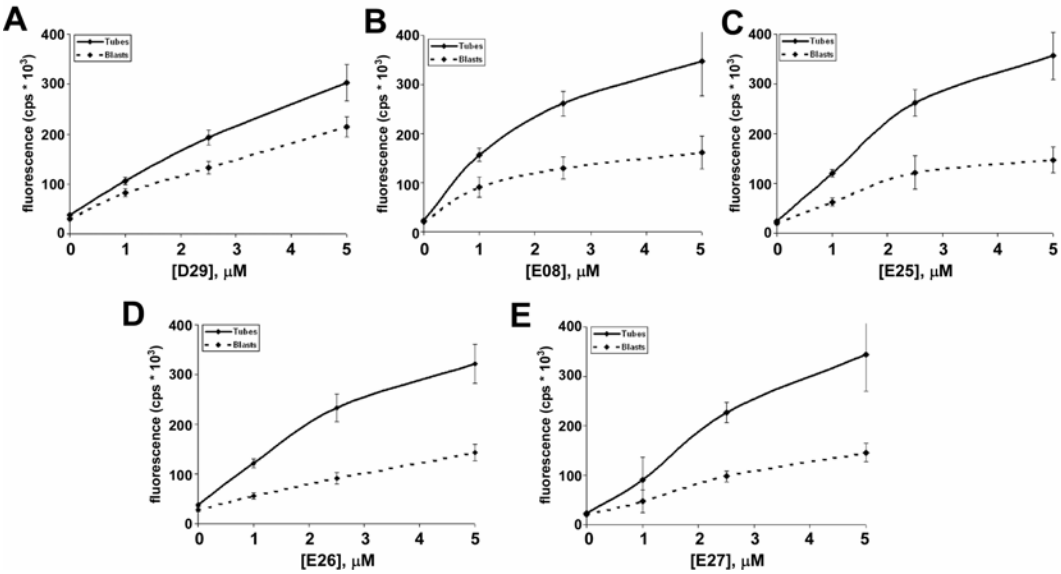

Figure S3. High-throughput dose-response analysis for rosamine fluorescence in myoblasts and myotubes: **(A)** compound **D29**, **(B)** compound **E08**, **(C)** compound **E25**, **(D)** compound **E26**, and **(E)** compound **E27**. Plate-reader fluorescence intensities of myoblasts and myotubes are shown for each concentration of rosamine tested, and reflect the mean and standard deviation of 192 wells of a 384-well plate. Note that **E23** (not shown) displayed no selectively at 1 μ*M*, and was not pursued further.

As a result of plate-reader experiments, we decided to use **E26** as our final screening agent, because it provided the greatest difference in fluorescence intensity between myoblasts and myotubes at 1 μ*M*.

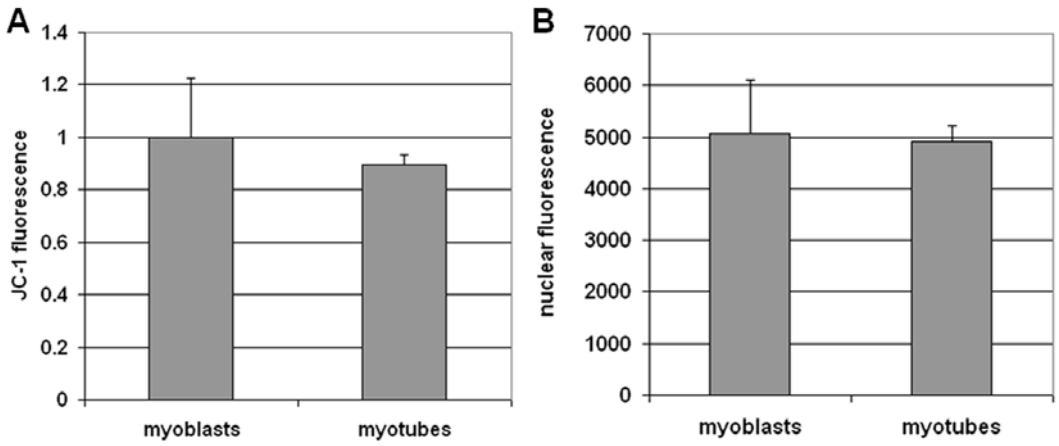

Figure S4. Determination of cellular parameters that could potentially correlate with cellular **E26** fluorescence. **(A)** Mitochondrial membrane potential was measured in myoblasts and myotubes using the JC-1 dye. Data shown use myoblasts as the baseline condition, and are the results of 48 wells of a 384-well plate per cellular condition. **(B)** Nuclei were stained with Hoechst 33342, and imaged and quantified using an ImageXpress Micro automated microscope and quantified using MetaXpress. Data shown are the results of 26 wells of a 384-well plate per cellular condition.

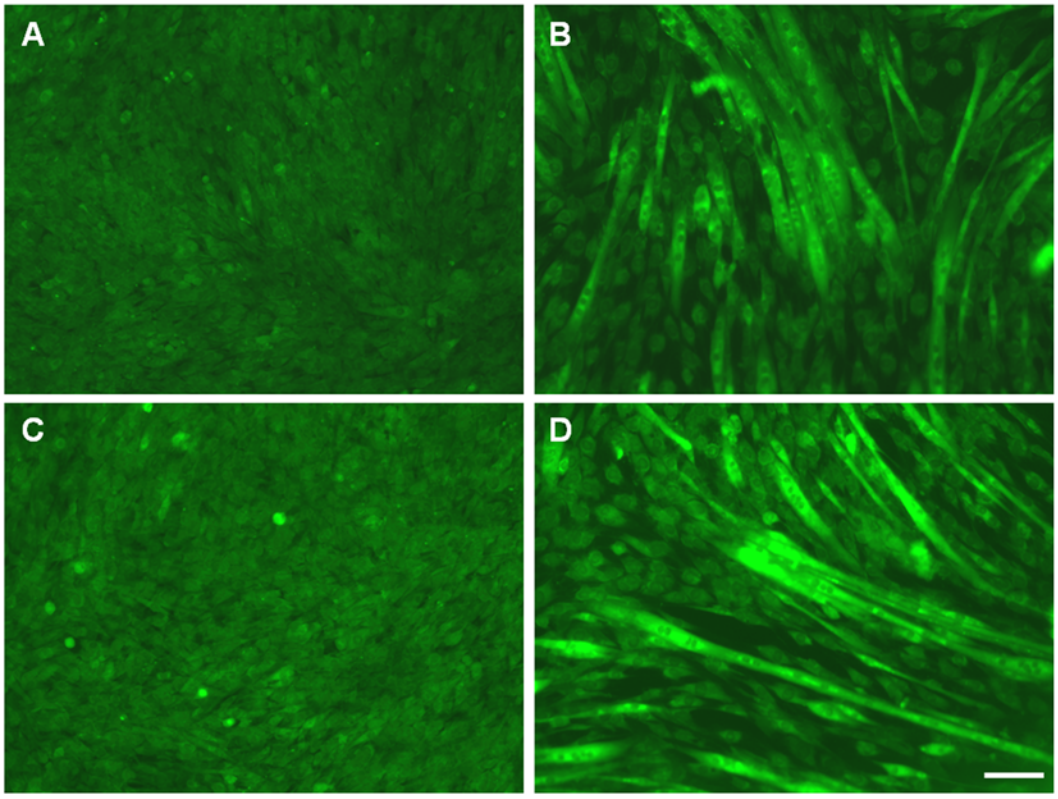

Figure S5. Assessment of solvent dependence on **E26** fluorescence. **(A)** C2C12 myoblasts and **(B)** myotubes fixed with 3.7% formaldehyde and then stained with **E26** diluted in phenol red-free DMEM cell-culture media. **(C)** C2C12 myoblasts and **(D)** myotubes fixed with 3.7% formaldehyde and then stained with **E26** diluted in PBS. Scale bar = 100 μm.

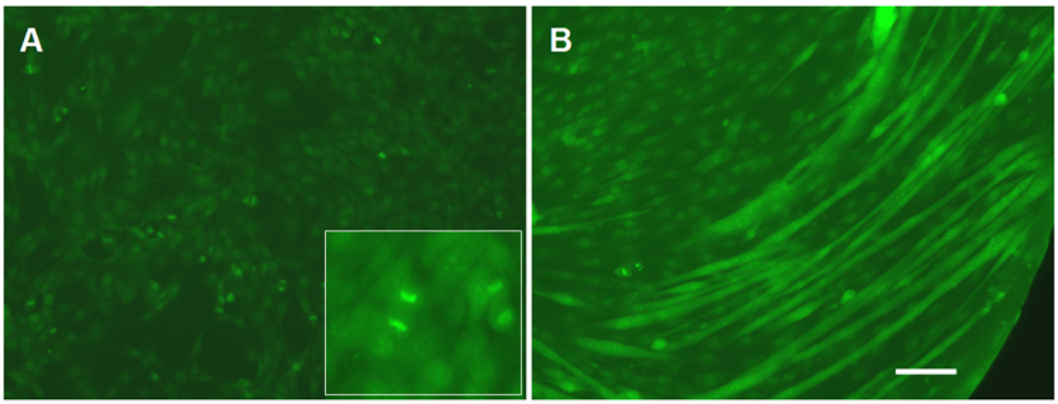

Figure S6. Assessment of detergent effects on **E26** fluorescence. **(A)** C2C12 myoblasts and **(B)** myotubes fixed with 3.7% formaldehyde and extracted with 0.5% Triton X-100 in PBS for 10 minutes before staining with **E26**. Scale bar = 100 μm. Inset in (A) shows higher magnification image of detergent-extracted myoblasts stained with E26, revealing enhanced staining of the separating chromosomes of a dividing cell.

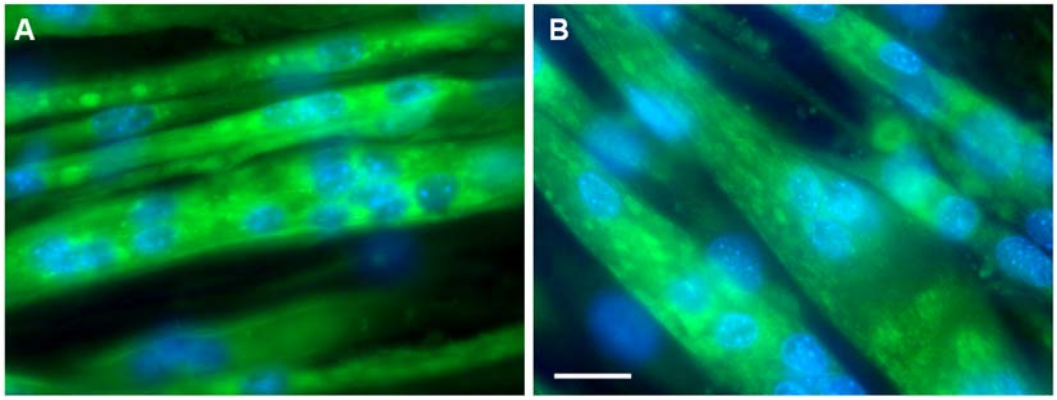

Figure S7. High-magnification images of **E26** fluorescence in C2C12 myotubes to assess cellular structure of fluorescence localization. **(A)** Myotubes, fixed with 3.7% formaldehyde before staining with **E26**. **(B)** Live fluorescence microscopy of myotubes stained with **E26**. Nuclei were stained with Hoechst 33342 before imaging. Scale bar = 20 μm.

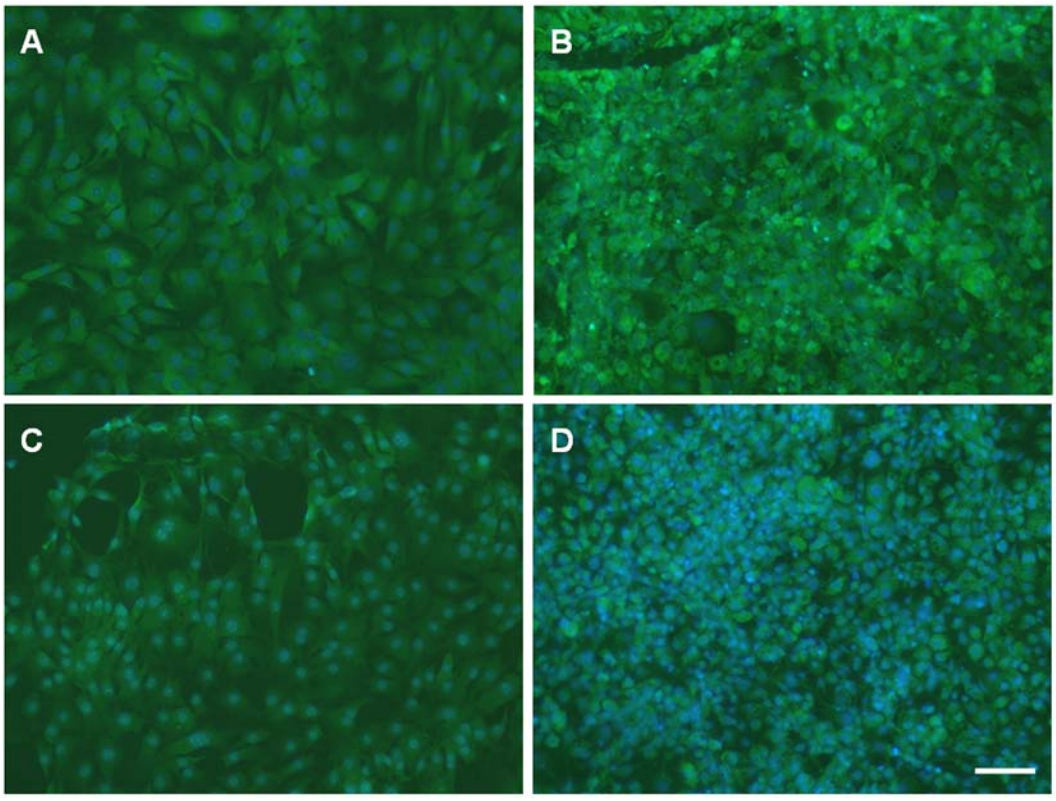

Figure S8. Testing E26 fluorescence in another model of cellular differentiation, 3T3-L1 adipogenesis. **(A)** Preadipocytes and **(B)** adipocytes, fixed with 3.7% formaldehyde before staining. **(C)** Preadipocytes and **(D)** adipocytes, fixed with 100% methanol before staining. Nuclei were stained with Hoechst 33342 prior to microscopy. Scale bar = 100 μm.

4. High-throughput screening for myogenesis

Quantitative data for high-throughput screening of the kinase inhibitor collection for inhibition of myogenesis is included in Table S4. Note that the rapamycin concentration shown in Figure 2 was higher when used for staining by **E26** (600 n*M*) than that used for MHC immunofluorescence (100 n*M*). This comparison was intended to be qualitative, and further studies are needed to quantify the relationship between **E26** staining and MHC immunofluorescence.

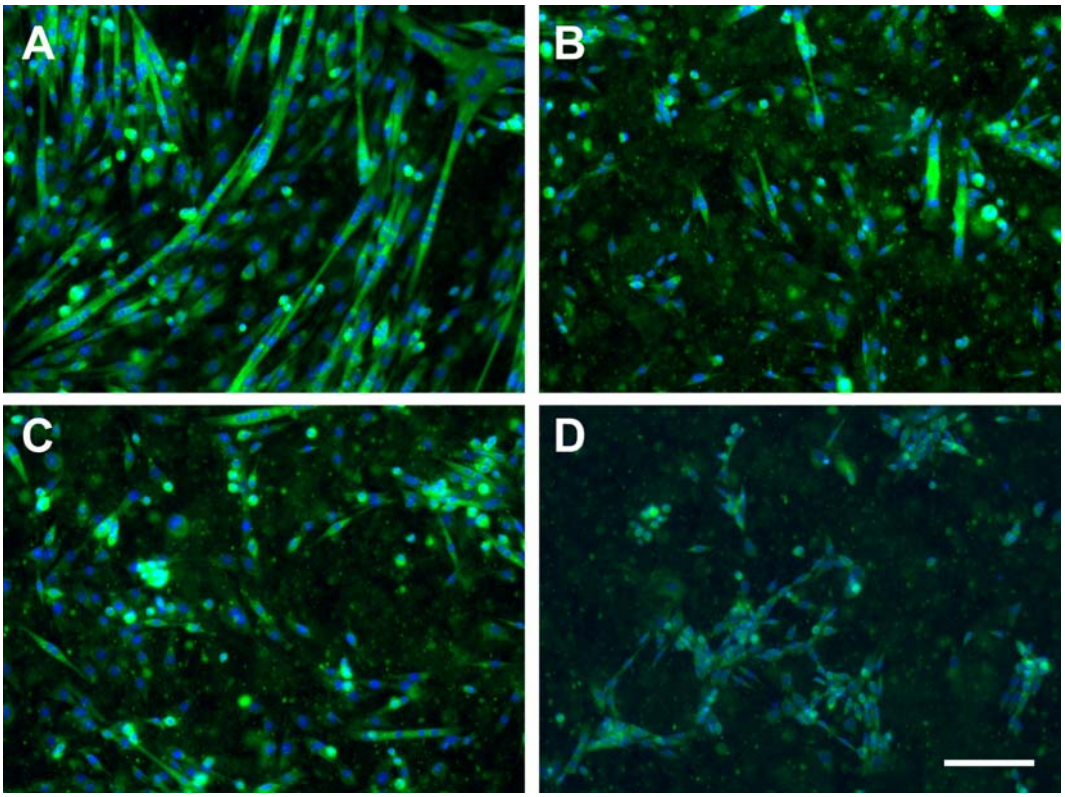

Figure S9. Representative two-color overlay images from myogenesis screening experiments. Cells were stained with 1 μ*M* **E26** (green), and DNA with Hoechst 33342 (blue). **(A)** Representative mock-treated (DMSO) well, **(B)** 2 μ*M* iodotubericidin, a MAP kinase inhibitor, **(C)** 600 n*M* wortmannin, a phosphatidylinositol 3-kinase inhibitor, **(D)** 600 n*M* rapamycin, an mTOR inhibitor. Scale bar = 150 μm.

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