



Figures and figure supplements

Myogenic regulatory transcription factors regulate growth in rhabdomyosarcoma

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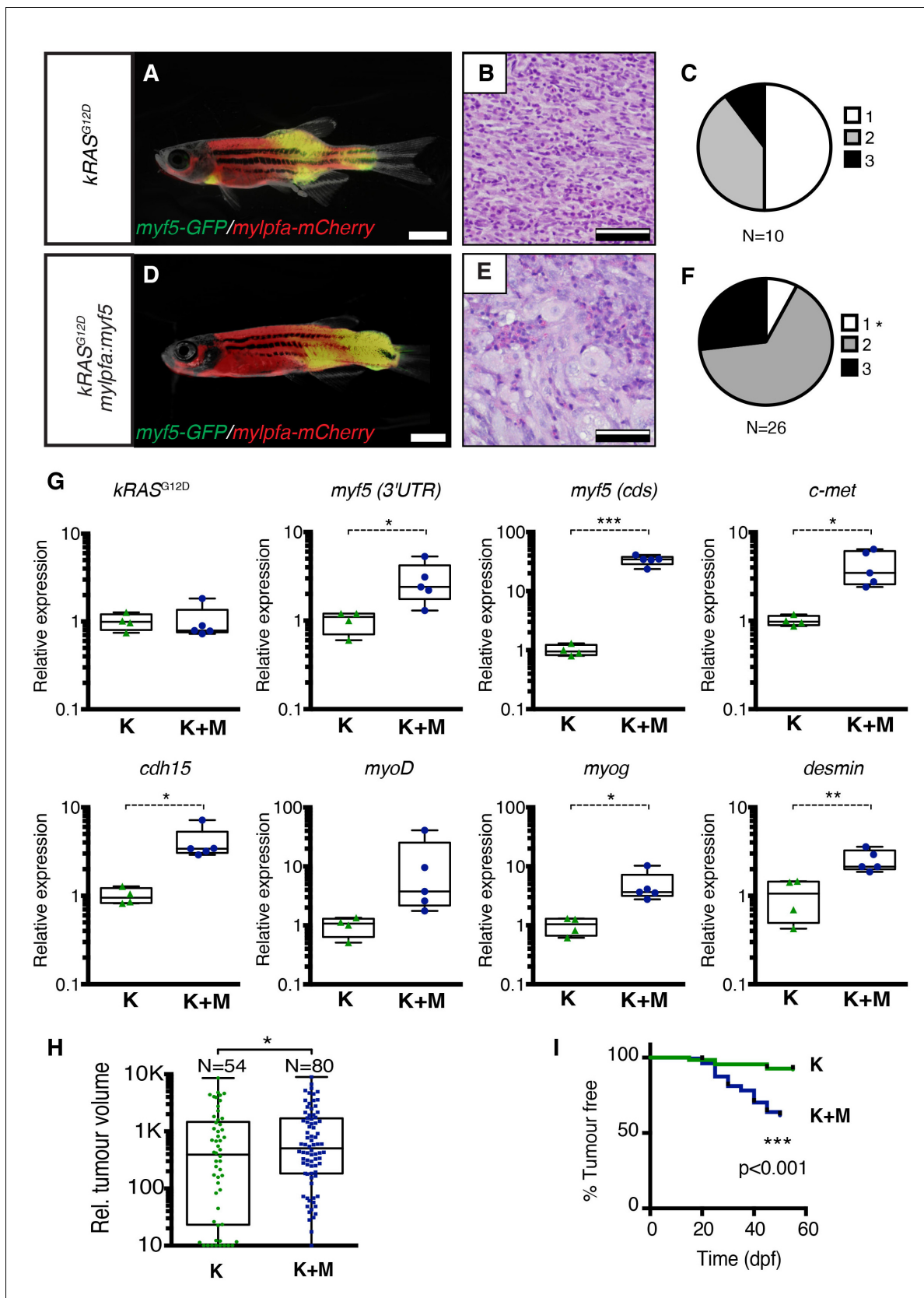


Figure 1. Transgenic *myf5* elevates tumor cell differentiation, increases tumor size, and accelerates time to primary tumor-onset when expressed in myosin-expressing ERMS cells. (A–F) Primary ERMS developing in *myf5:GFP/mylpfa:mCherry* AB-strain zebrafish. Transgenic *kRAS^{G12D}*-expressing

Figure 1 continued on next page

Figure 1 continued

ERMS (A–C) compared with those that express both *kRAS*^{G12D} and *mylpfa:myf5* (D–F). Animals imaged at 35 dpf (A,D). Hematoxylin and Eosin-stained sections of representative tumors (B,E) and quantification of differentiation within individual tumors (C,F; 1-less differentiated and 3-most differentiated). Asterisk denotes $p=0.015$ by Chi-square test. (G) Quantitative real-time PCR gene expression performed on bulk ERMS cells, confirming high *myf5* expression, increased differentiation, and high expression of TPC associated genes in ERMS that co-express *kRAS*^{G12D} and *mylpfa:myf5* (K+M, N = 5). Endogenous *myf5* was assessed using primers specific to the 3'UTR and total *myf5* assessed by primers that amplify the coding sequence (cds). *cadherin 15* (*cdh15*) and *myogenin* (*myog*). *kRAS*^{G12D} alone expressing ERMS (K, N = 4). Average gene expression with 50% confidence intervals denoted by box. Mean, maximum, and minimum also denoted. (H) Relative tumor size of primary ERMS at 30 days post fertilization (dpf). Box shows 50% confidence interval. Mean, maximum, and minimum denoted. Asterisk denotes $p=0.0108$, Student's t-test. (I) Kaplan-Meijer analysis denoting time-to-tumor onset ($p<0.001$, Log-rank Statistic, N = 494 fish analyzed for K and N = 470 for K+M). Scale bars equal 2 mm (A,D) and 50 μ m (B,E). Asterisks in panels G–H denote * $p<0.05$; ** $p<0.01$; *** $p<0.001$ by Student's t-test.

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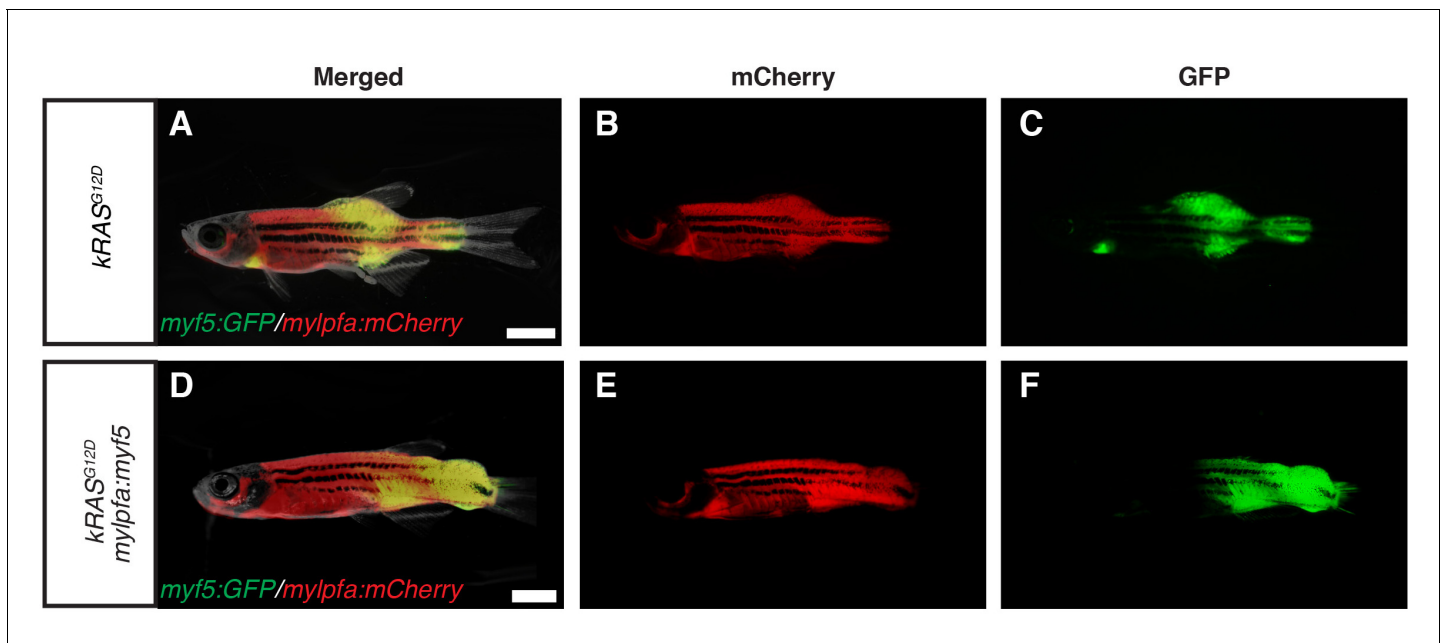


Figure 1—figure supplement 1. Fluorescence images of primary ERMS developing in stable transgenic *myf5:GFP/mylpfa:mCherry* zebrafish. Images of the same representative *rag2:kRAS^{G12D}*—alone (A–C) and *rag2:kRAS^{G12D}; mylpfa:myf5* (D–F) zebrafish shown in **Figure 1A and D**, respectively. (A,D) merged (brightfield, GFP and mCherry) image. (B,E) mCherry image. (C,F) GFP image. Scale bars equal 2 mm.

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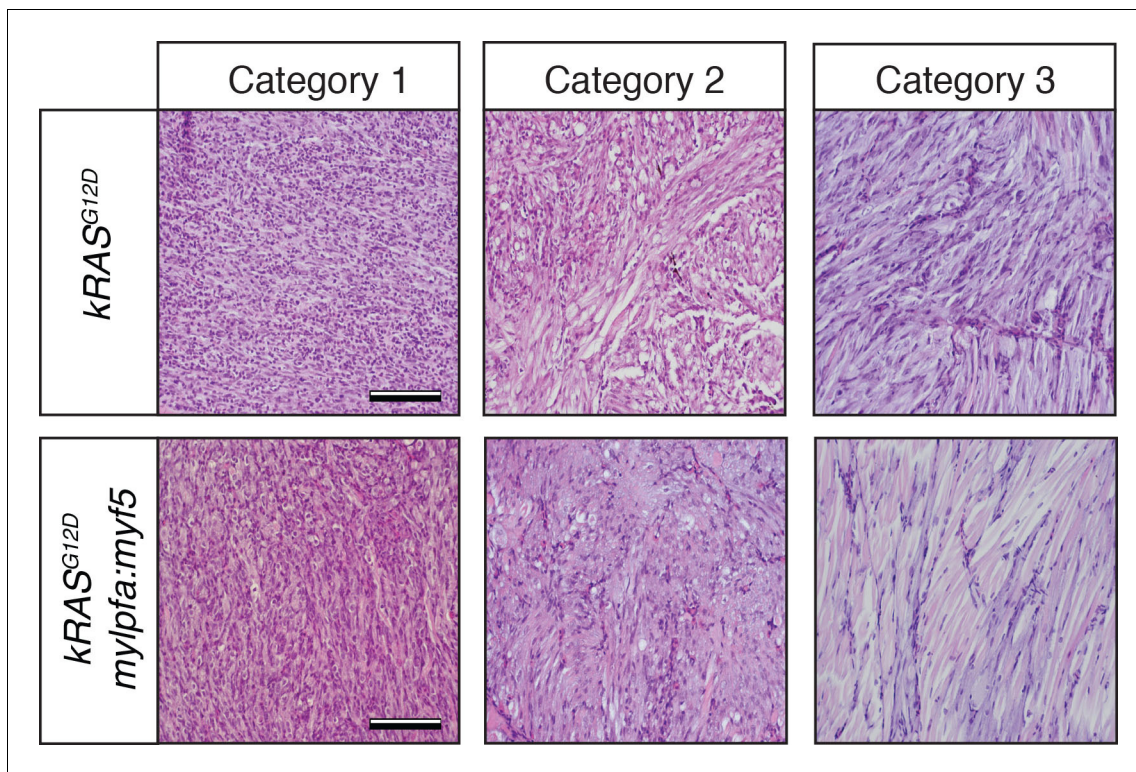


Figure 1—figure supplement 2. Histological classification of primary zebrafish ERMS based on differentiation score. Representative H and E-stained sections of zebrafish ERMS assigned to each differentiation category. Scale bars equal 100 μm .

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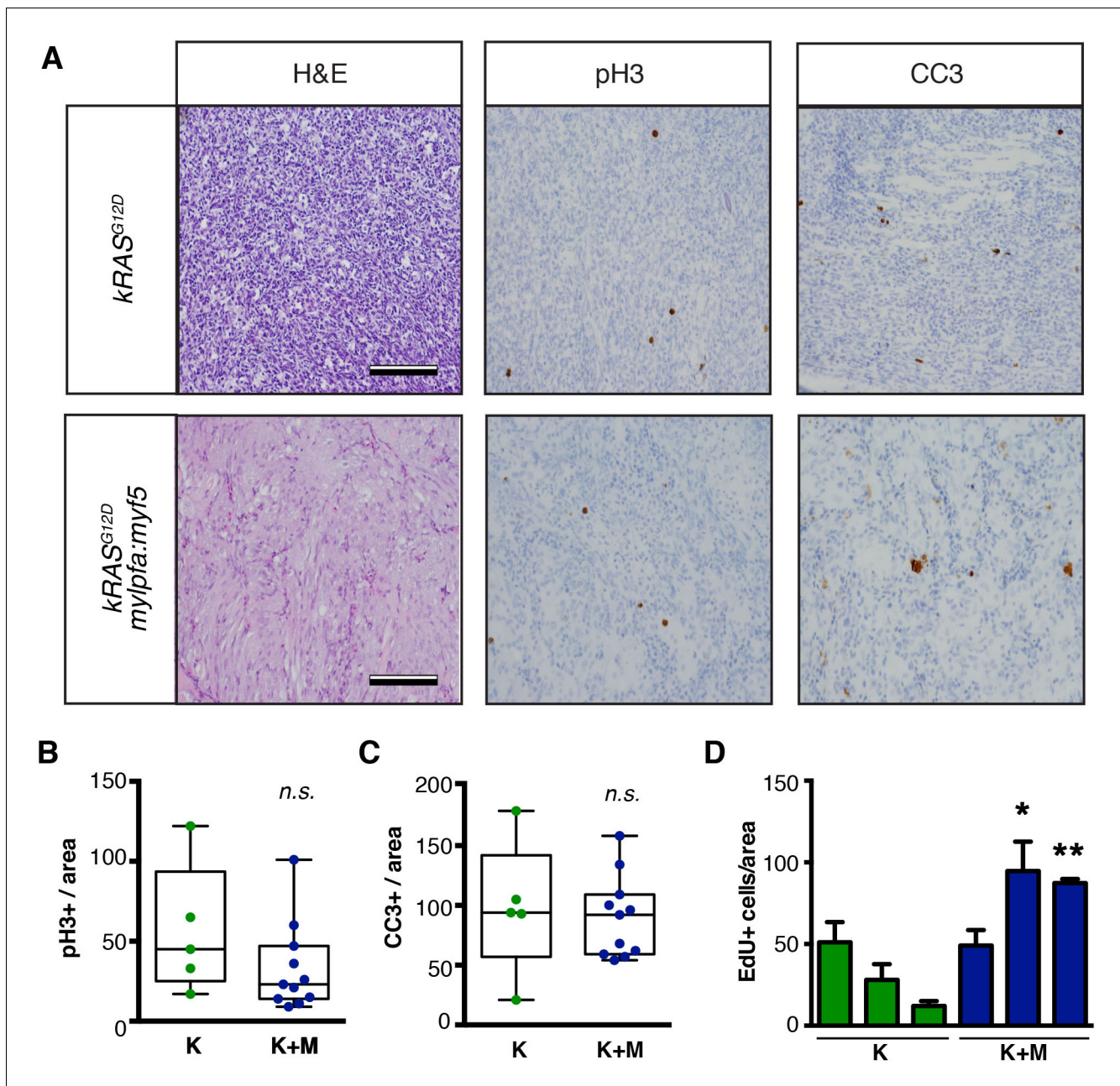


Figure 1—figure supplement 3. Analysis of proliferation and apoptosis in zebrafish primary ERMS. (A) Representative H and E-stained sections and immunohistochemistry for phospho-H3 (pH3) and cleaved caspase-3 (CC3). (B) Quantification of the total number of pH3-positive cells per 400x imaging field. (n=average of 3 fields/tumor section). (C) Quantification of the total number of CC3-positive cells per 400x imaging field (n=average of 3 fields/tumor). Boxes in B–C denote 50% confidence interval and mean, maximum, and minimum shown. *kRAS^{G12D}*[K] (N = 5) and *kRAS^{G12D}; mylpfa:myf5* [K+M] (N = 11). (D) Quantification of total number of EdU+ cells per area (n=average of 3 fields/tumor. N = 3 tumors per genotype). *p<0.05 or **p<0.01 in comparison to each *kRAS^{G12D}*-alone expressing ERMS (Student's t-test). Error bars denote +/- STD. Scale bars equal 100 μ m (A). Not significant by Student's t-test (*n.s.*).

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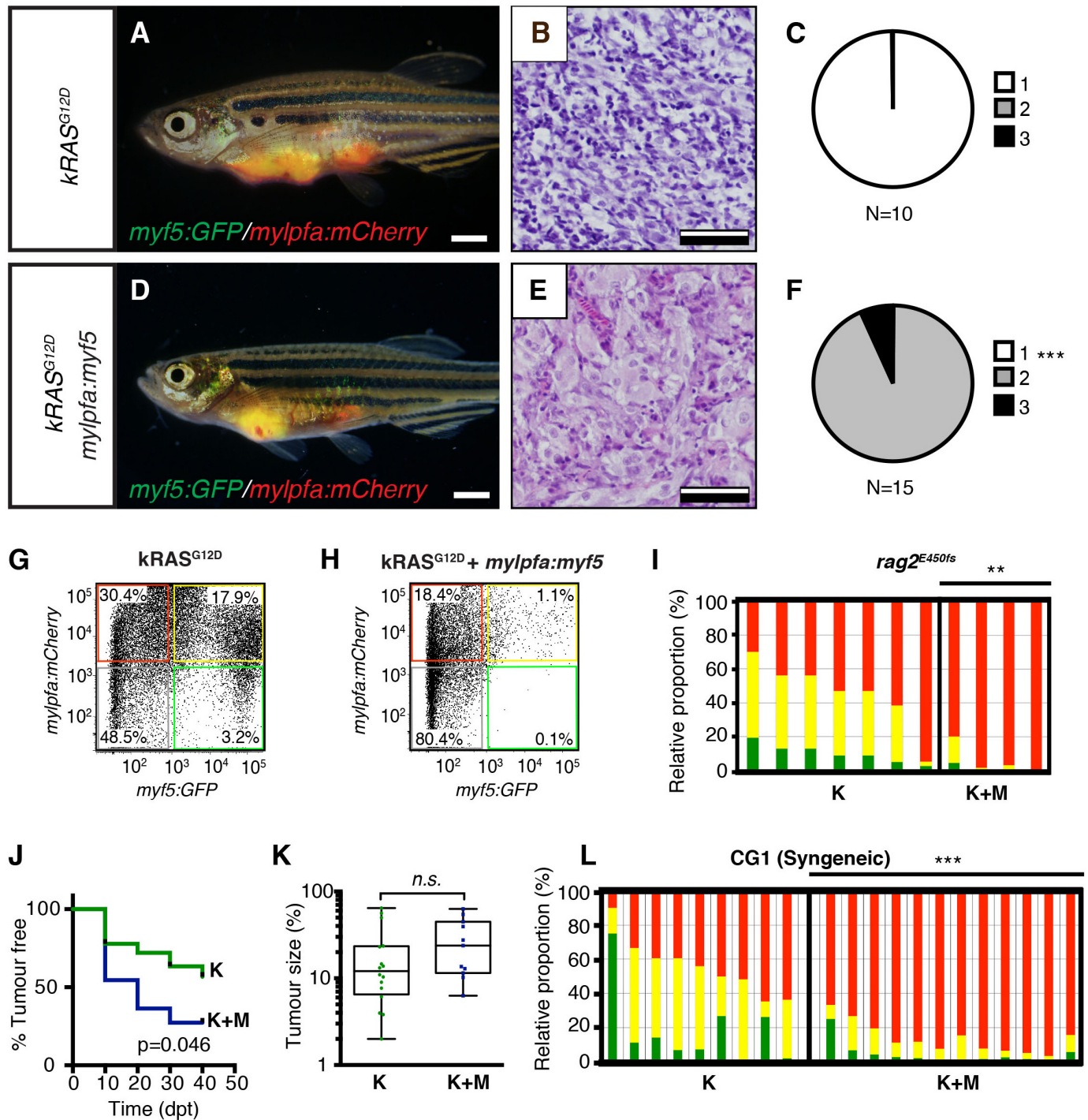


Figure 2. Tumors that transgenically express *myf5* are fully transformed and retain a differentiated phenotype following engraftment into recipient animals. (A–F) Analysis of ERMS arising in transplanted fish. *kRAS*^{G12D} expressing ERMS arising in *rag2*^{E450fs} transplant recipient fish (A–C) compared with those that express both *kRAS*^{G12D} and *mylpfa:myf5* (D–F). Tumors were created in stable transgenic *myf5:GFP/mylpfa:mCherry* transgenic, AB-strain zebrafish and imaged following engraftment into recipient fish at 30 days post transplantation (dpt). Hematoxylin and eosin stained sections of representative tumors (B,E) and quantification of differentiation within individual ERMS (C,F; 1-less differentiated and 3-most differentiated). Asterisks denote p < 0.001 by Chi-square test. (G,H) Representative flow cytometry analysis of fluorescently-labeled ERMS cells isolated from transplanted *rag2*^{E450fs} zebrafish. (I) Graphical summary of ERMS cell sub-fractions that grow following engraftment into immune-deficient *rag2*^{E450fs} recipients. Figure 2 continued on next page

Figure 2 continued

Individual tumors are represented as separate bars with the proportion of G+ (green), G+R+ (yellow) and R+ (red) sub-populations denoted. **p=0.006. (J) Kaplan-Meijer analysis showing time-to-tumor onset in transplanted ERMS arising in *rag2^{E450fs}* zebrafish (p=0.046, Log-rank Statistic, 2×10^5 cells/fish, N > 12 animals per arm, representing ≥ 3 independently-arising primary ERMS). (K) Relative tumor size at 30 days post engraftment (same animals analyzed as in J). (L) ERMS cells were also more differentiated following engraftment of *myf5:GFP/mylpfa:mCherry* ERMS cells into syngeneic recipient fish (p<0.001, Student's T-test, N ≥ 3 independently arising primary ERMS and assessed in n ≥ 2 animals per transplanted tumor). Scale bars equal 2 mm (A,D) and 50 μ m (B,E).

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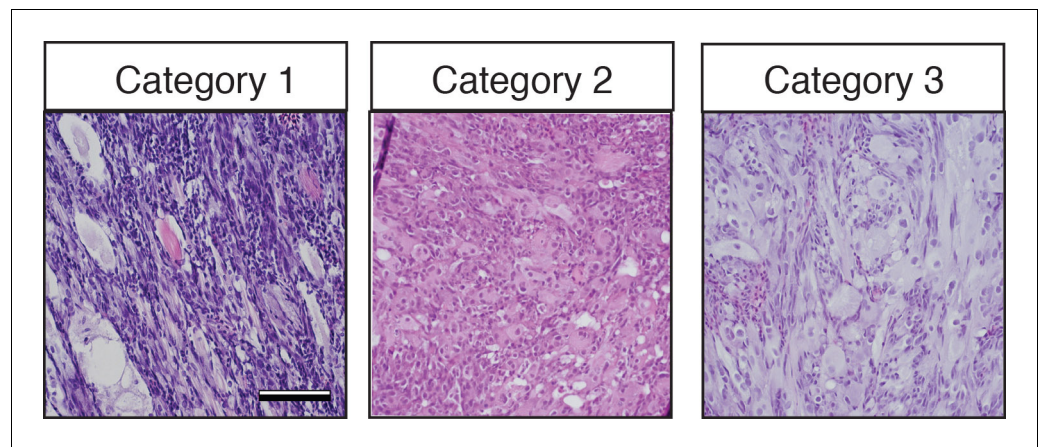


Figure 2—figure supplement 1. Histological classification of transplanted zebrafish ERMS based on differentiation score. Representative H and E-stained sections of zebrafish ERMS assigned to each differentiation category. Scale bars equal 100 μ m.

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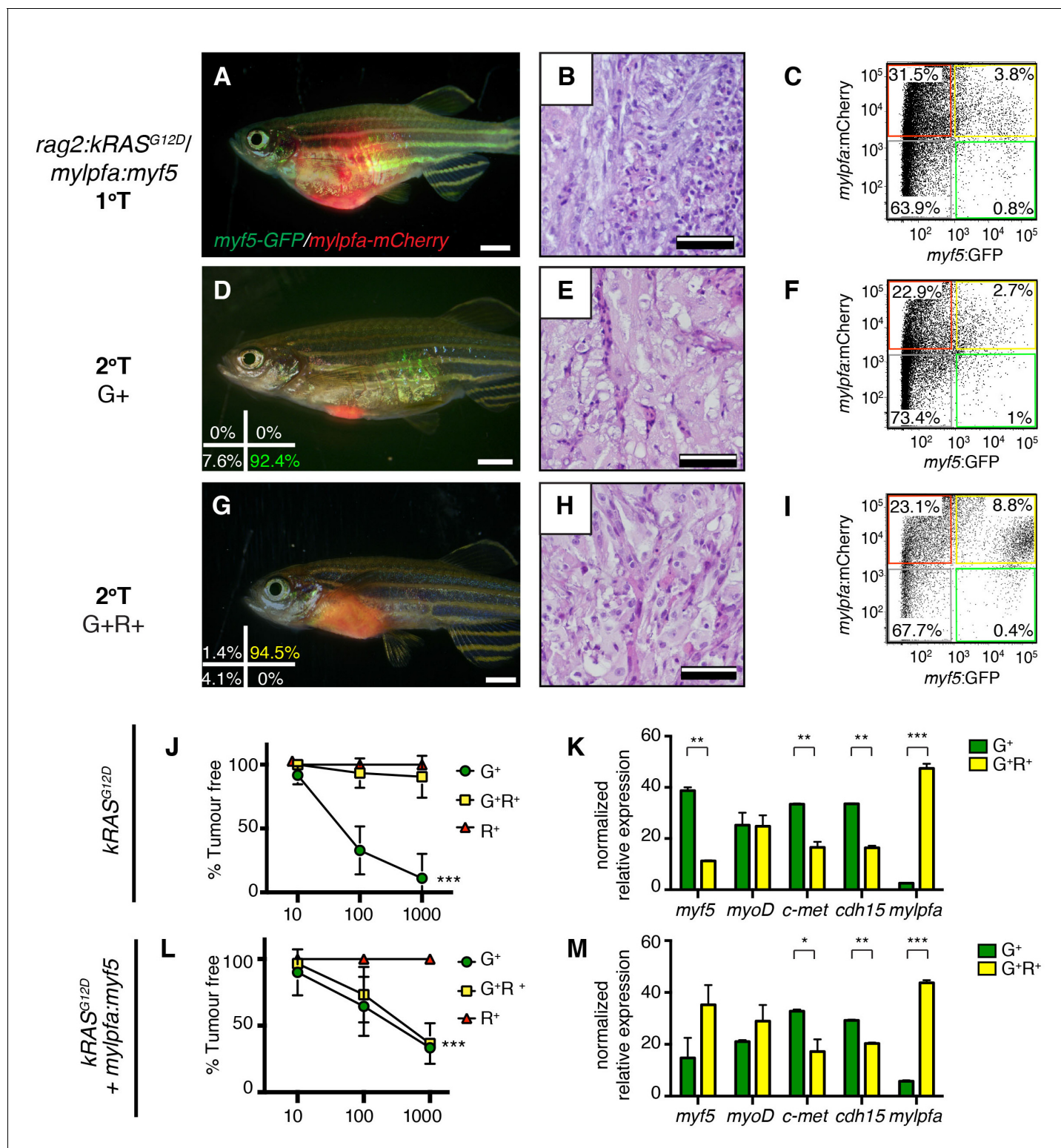


Figure 3. Limiting dilution cell transplantation shows that *myf5* can confer tumor-propagating ability to differentiated *myf5:GFP*⁺/*mylpfa:mCherry*⁺ cells. Tumors were generated in *myf5:GFP/mylpfa:mCherry* CG1-strain syngeneic zebrafish. Representative tumors arising in primary transplanted fish (1°T, A–C) or secondary transplanted fish following engraftment with highly purified *myf5:GFP*⁺, *mylpfa:mCherry*-negative (2°T G⁺, D–F) or *myf5:GFP*⁺, *mylpfa:mCherry*⁺ ERMS cells (2°T G+R⁺, G–I). Sort purity following FACS is noted in the lower left panels of D and G and was >92% for each population. These cells were used for cell transplantations and data provided in D–I. Cell viability was >95%. (J,L) Graphical summary of tumor engraftment following limiting dilution cell transplantation using highly purified sorted ERMS cells. Data is combined from all tumors shown in **Table 1**. Figure 3 continued on next page

Figure 3 continued

*** $p < 0.0002$ by ELDA analysis. (K,M) Relative gene expression analysis of sorted G+ or G+R+ ERMS cells from representative $kRAS^{G12D}$ (K) or $kRAS^{G12D}; mylpfa:myf5$ (M) expressing ERMS (Standard Deviation, $n = 3$ technical replicates per PCR condition). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

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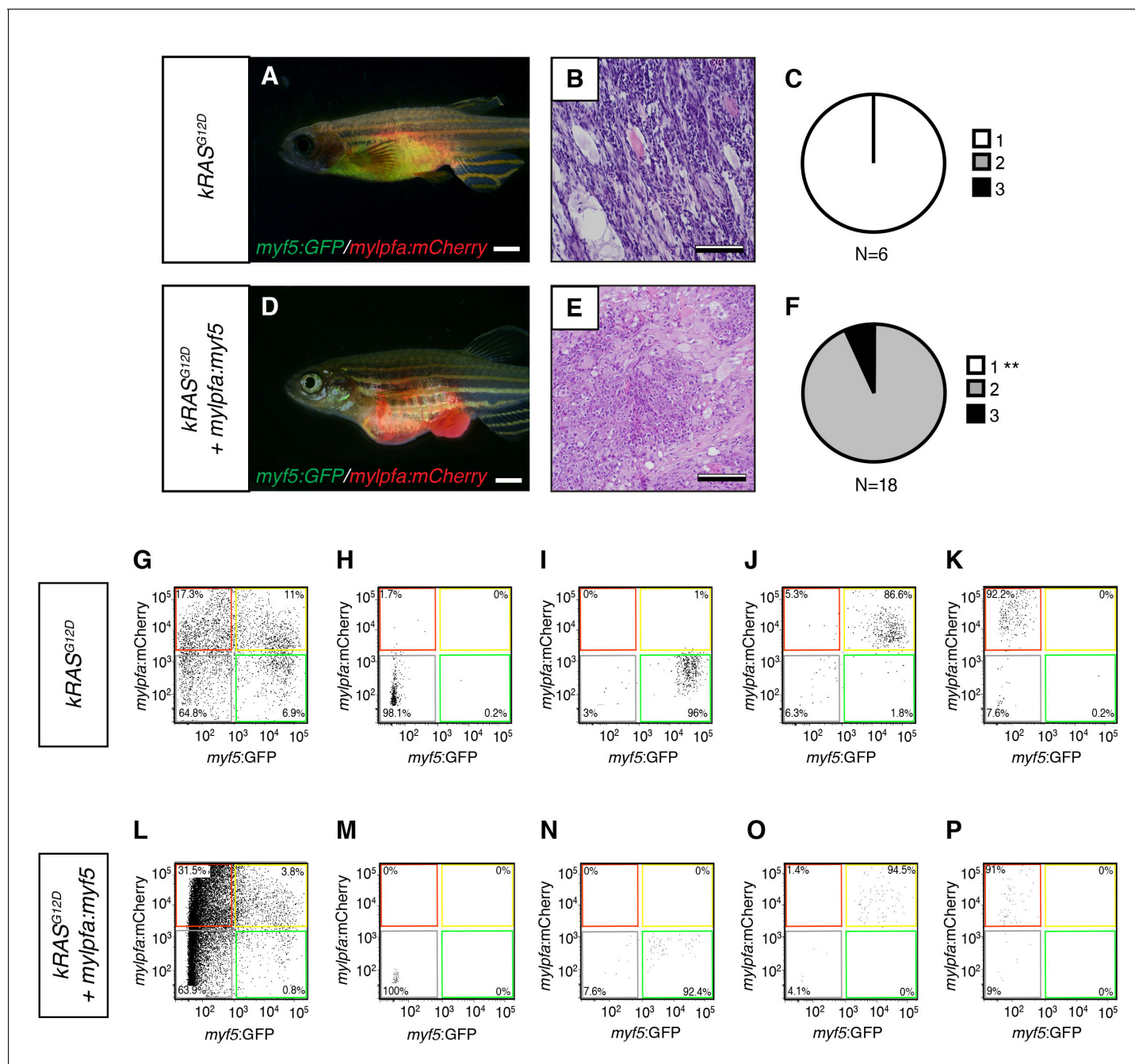


Figure 3—figure supplement 1. Analysis of transplanted ERMS arising in CG1-strain syngeneic recipients. (A,D) Representative images of transplanted fish. ERMS were created in $myf5:GFP/mylpfa-mCherry$ transgenic, CG1-strain syngeneic zebrafish and imaged following 30 days of engraftment. Genotypes denoted to the left. (B,E) Representative histology of transplanted tumors. (C,F) Quantification of differentiation based on histological review (1-less differentiated and 3-most differentiated). ** $p < 0.01$ by Chi-square test. (G–P) Representative examples of sort purity following FACS for cells used in limiting dilution cell transplantation experiments. (G–K) Sort purity following FACS for a representative $kRAS^{G12D}$ -alone expressing ERMS and (L–P) $kRAS^{G12D} + mylpfa:myf5$ expressing ERMS (data is reproduced in lower left panels of **Figure 3D and G**). Scale bars equal 2 mm (A,D) and 100 μm (B,E).

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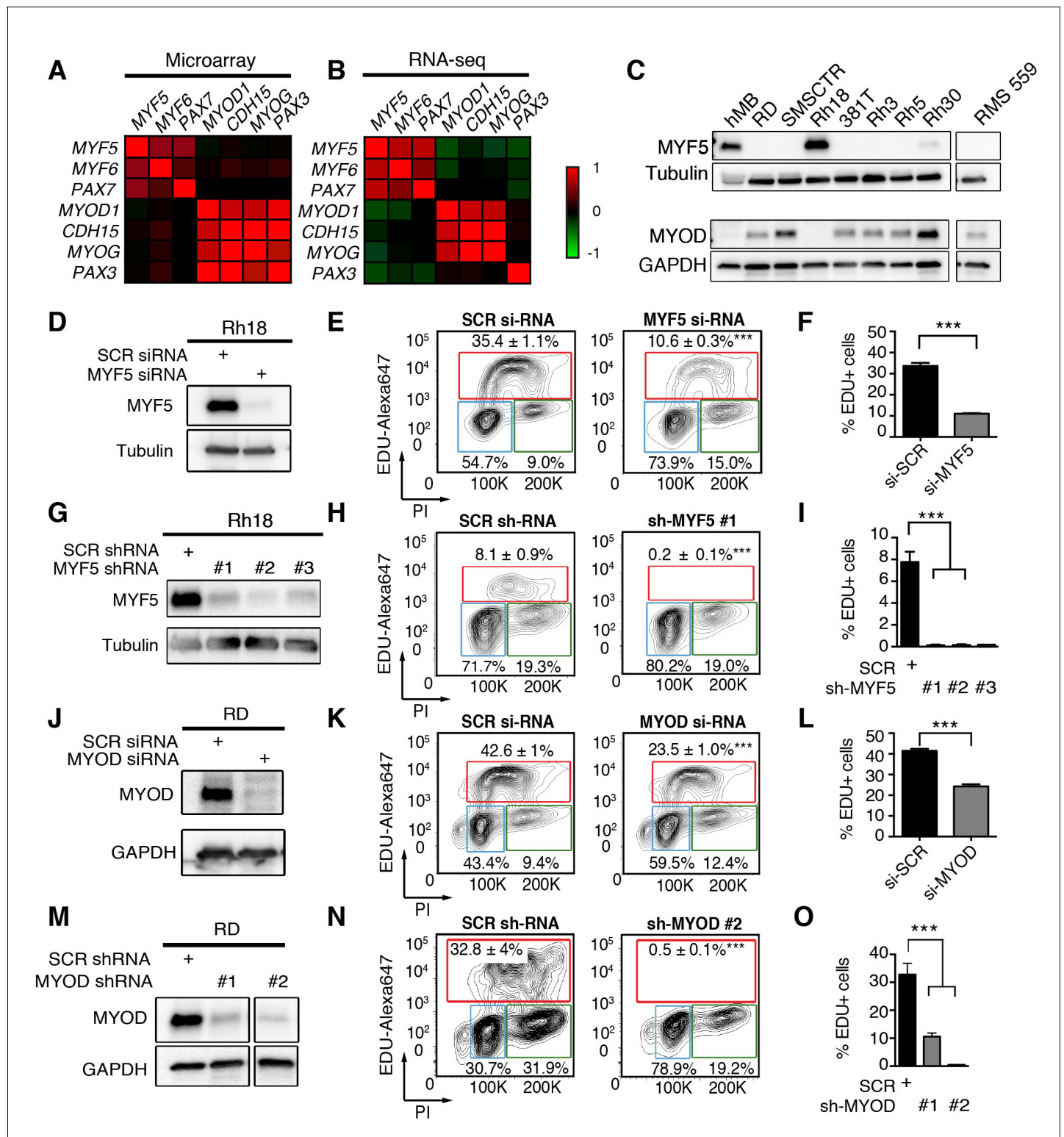


Figure 4. MYF5 and MYOD are required for human ERMS proliferation and growth. (A–B) Pearson correlation for gene expression of myogenic genes in primary human RMS as assessed by microarray (A) or RNA-sequencing (B). Heatmap represents correlation coefficients. (C) Western blot analysis for MYF5 and MYOD in human RMS cell lines. (D–I) Rh18 ERMS cells following MYF5 knockdown with siRNA (D–F) or shRNA (G–I). (J–O) RD ERMS cells following MYOD knockdown with siRNA (J–L) or shRNA (M–O). Western blot analysis following knockdown at 48 hr (D,J) and 72 hr (G,M). EdU and Propidium Iodide (PI) cell cycle analysis assessed by flow cytometry at 48 hr (E,F,K,L) and 72 hr (H,I,N,O). Standard Deviation denoted in FACS plots

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Figure 4 continued

and graphs. Analysis shown in D-O was completed as technical replicates and completed ≥ 3 independent times with similar results. *** $p < 0.001$ by Student's t-test.

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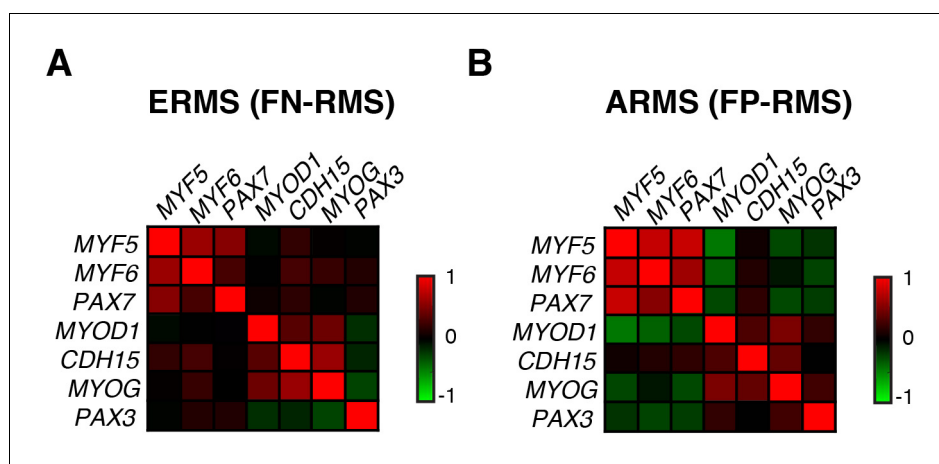


Figure 4—figure supplement 1. Pearson correlation of gene expression from RNA-seq data of primary human RMS. (A) Analysis of RNA-seq data from primary fusion-negative RMS (FN-RMS; N = 70) and (B) fusion-positive RMS (FP-RMS; N = 33 samples).

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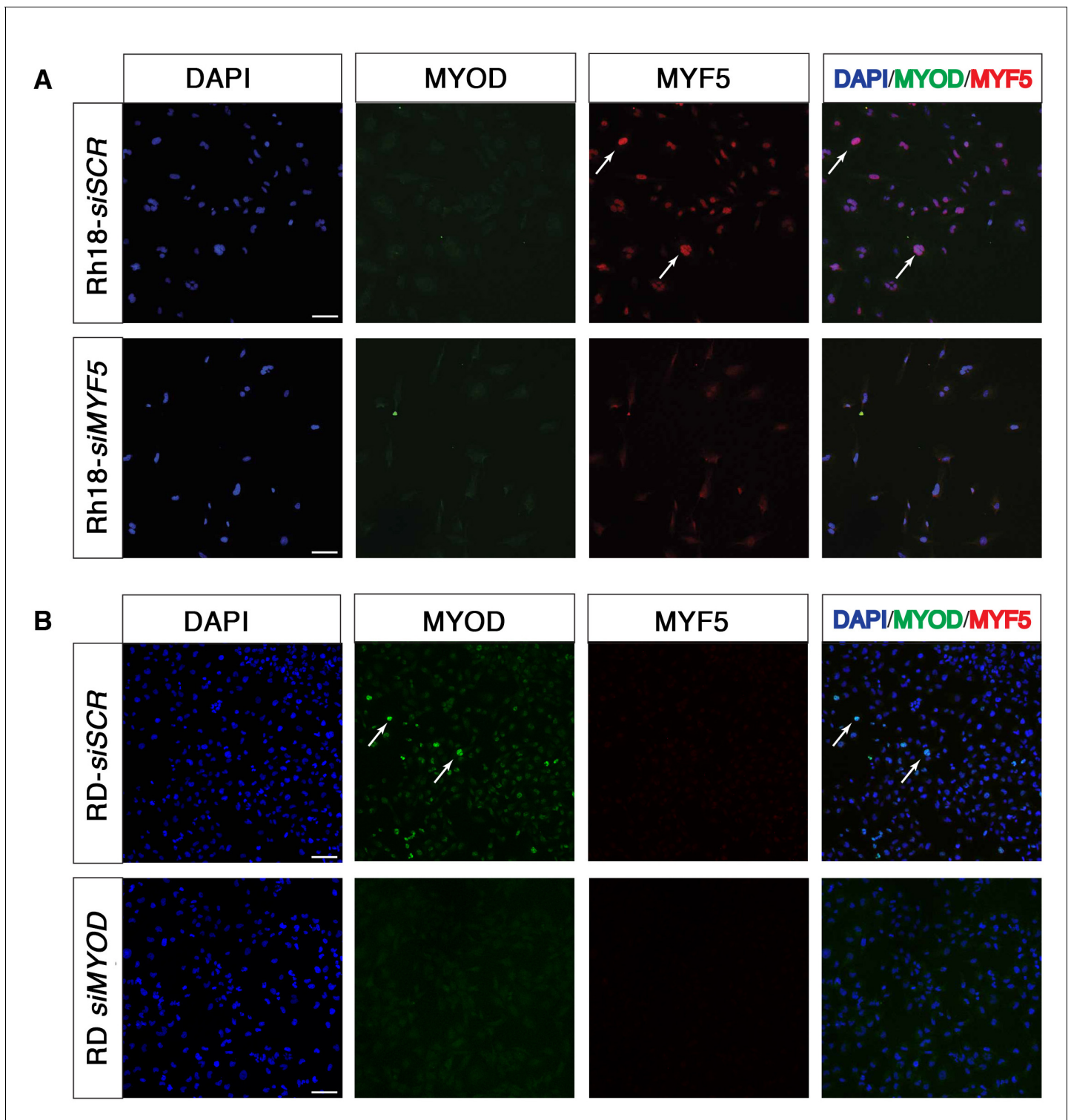


Figure 4—figure supplement 2. Immunofluorescence for MYF5 and MYOD in Rh18 and RD ERMS cell lines. (A) Confocal microscopy images of DAPI and antibody immunofluorescence-staining of Rh18 cells treated with control siRNA or si-MYF5 for 72 hr. (B) Confocal microscopy images of DAPI and antibody immunofluorescence staining of RD cells treated with control siRNA or si-MYOD. Anti-MYOD (green) and anti-MYF5 (red) and counterstained with DAPI (blue). Merged image shown to right. Scale bar equals 100 μ m. Arrows denote representative examples of MYF5+/MYOD-negative RH18 cells in (A) and MYF5-negative/MYOD+ RD cells in (B).

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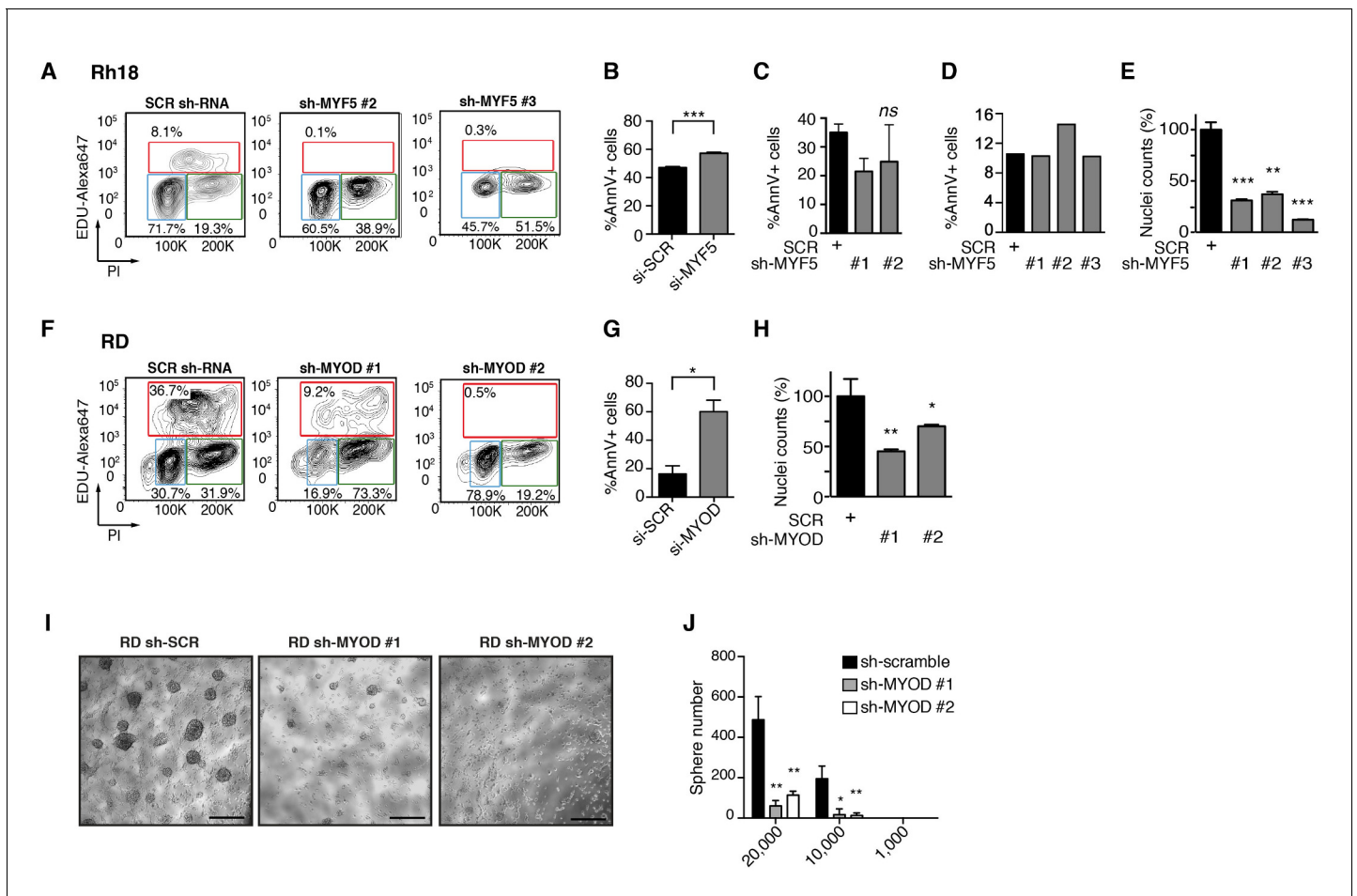


Figure 4—figure supplement 3. MYF5 and MYOD are required for human RMS proliferation and growth in vitro. (A–E) Rh18 ERMS cells following MYF5 knockdown and (F–J) RD ERMS cells following MYOD knockdown. EdU/PI Flow cytometry analysis performed at 72 hr post transfection with shRNAs (A,F). AnnV Flow cytometry quantification performed at 96 hr post siRNA transfection (B,G) or shRNA transfection (C–D). Quantitation of nuclei counts performed on shRNA treated cells at 96 hr post infection (E,H). (I,J) Sphere colony formation assays performed in RD ERMS cells. Representative images of sh-SCR, sh-MYOD #1 and sh-MYOD #2 treated cells (I), images denote growth when seeding at 1×10^4 cells/well). Quantification of total spheres formed following seeding with different numbers of cells/well (J). Analysis was completed as technical replicates and completed ≥ 3 independent times with similar results. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's t-test. Scale bar equals 50 μ m.

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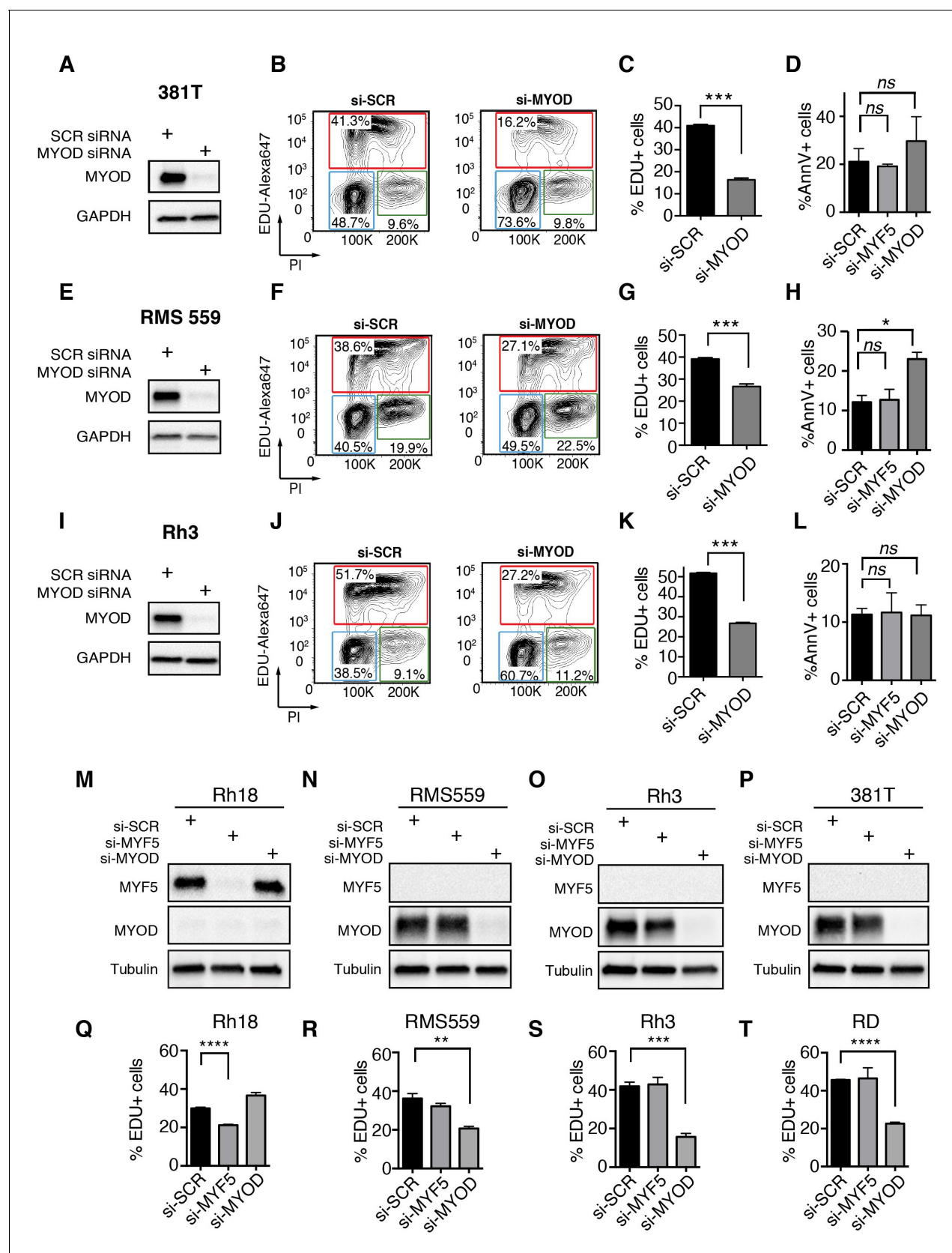


Figure 4—figure supplement 4. MYF5 and MYOD are each specifically required for human RMS proliferation and growth in vitro. (A–L) *siMYOD* knockdown effects in human RMS cell lines. (A–D) 381T ERMS; (E–H) RMS559 ERMS; (I–L) Rh3 ARMS/FP-RMS cells. (A,E,I) Western Blot analysis. Figure 4—figure supplement 4 continued on next page

Figure 4—figure supplement 4 continued

performed at 48 hr post siRNA transfection. (B,F,J) FACS plots for EdU/PI staining of cells at 48 hr after si-RNA transfection. (C,G,K) Quantification of EDU results. (D,H,L) AnnexinV Flow Cytometric analysis performed at 96 hr post transfection with *si-SCR* control, *si-MYF5* or *si-MYOD*. (M–T) MRF knockdown is specific to each expressed transcription factor. Western blot analysis of Rh18 (M), RMS559 (N), Rh3 (O) and 381T (P) cells following 48 hr of siRNA treatment. Quantitation of EdU/PI flow cytometric analysis for Rh18 (Q), RMS559 (R), Rh3 (S) and RD (T) cells following 48 hr of siRNA treatment. Knockdown effects for RD cells are shown in **Figure 6E**. Error bars denote \pm STD from three technical replicates. Experiments were replicated three times on different days, showing similar results. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ by Student's t-test. Not significant (n.s.). DOI: [10.7554/eLife.19214.015](https://doi.org/10.7554/eLife.19214.015)

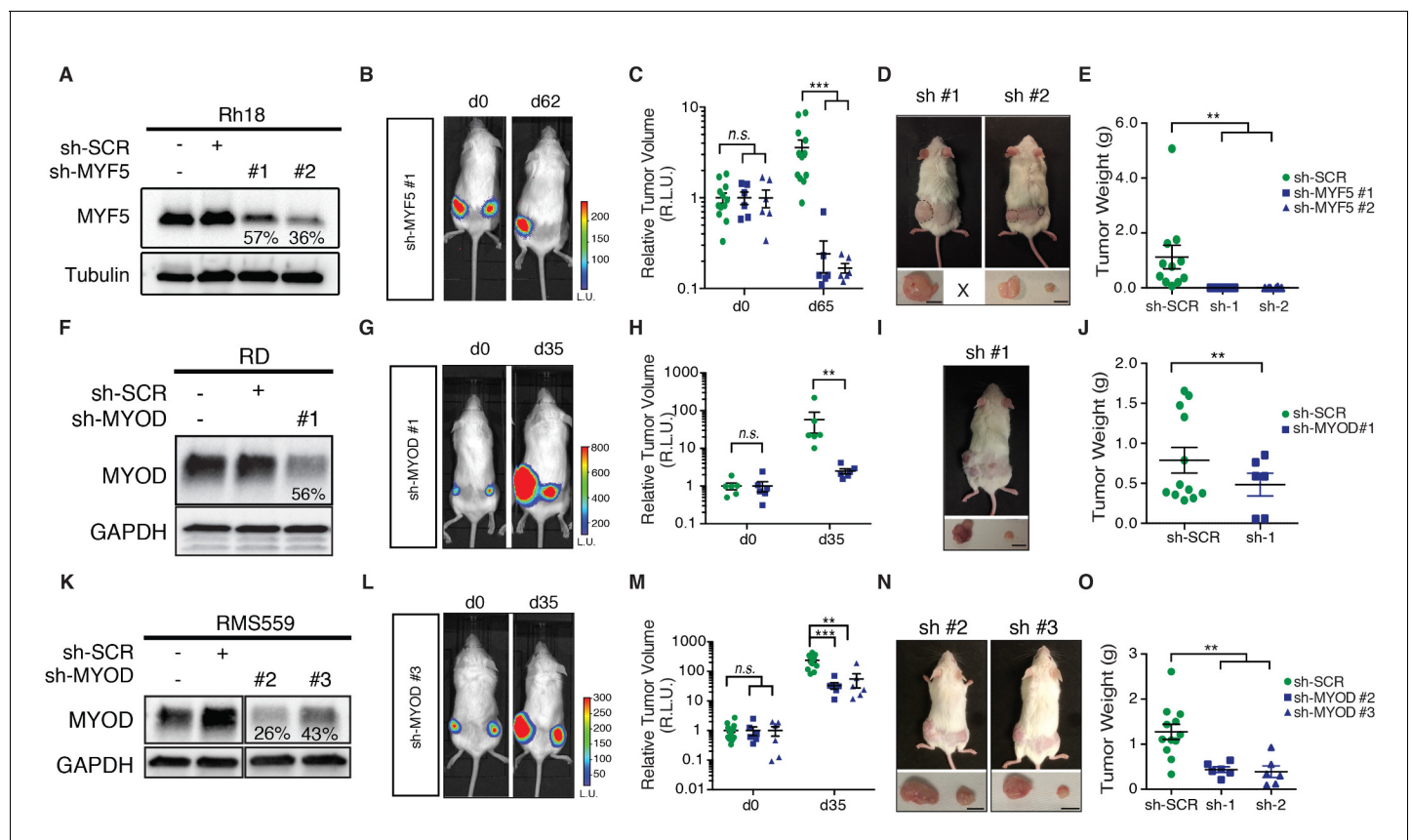


Figure 5. MYF5 and MYOD are required for human ERMS xenograft growth. Xenograft growth in Rh18 (A–E), RD (F–J), and RMS559 (K–O) following knockdown with scramble control shRNA (sh-SCR) or shRNAs specific to MYF5 or MYOD. (A,F,K) Western blot analysis of shRNA expressing cells harvested for transplantation at 72 hr after lenti-viral shRNA knockdown. Percent knockdown compared to shRNA control is shown. (B,G,L) Luciferase bioluminescent imaging of a representative animal at the time of implantation (left panel) or at later time points (right panel). Control shRNA cells were implanted into left flank and knockdown cells into the right (N = 6 mice per shRNA). Intensity represents total luminescence units measured per region of interest (L.U.) (C,H,M) Quantification tumor volume when assessed by luciferase imaging. Relative luminescence units (R.L.U.). (D,I,N) Representative images of mice at the time of necropsy, with excised tumors shown in lower panels. (E,J,O) Quantification of tumor weight at the time of necropsy. Tumors that could not be identified at time of necropsy were assigned a value of zero for this analysis. Standard Error of the Mean are denoted in graphs. **p<0.01; ***p<0.001 by Mann-Whitney non-parametric test. Scale bar equals 1 cm in D,I, and N.

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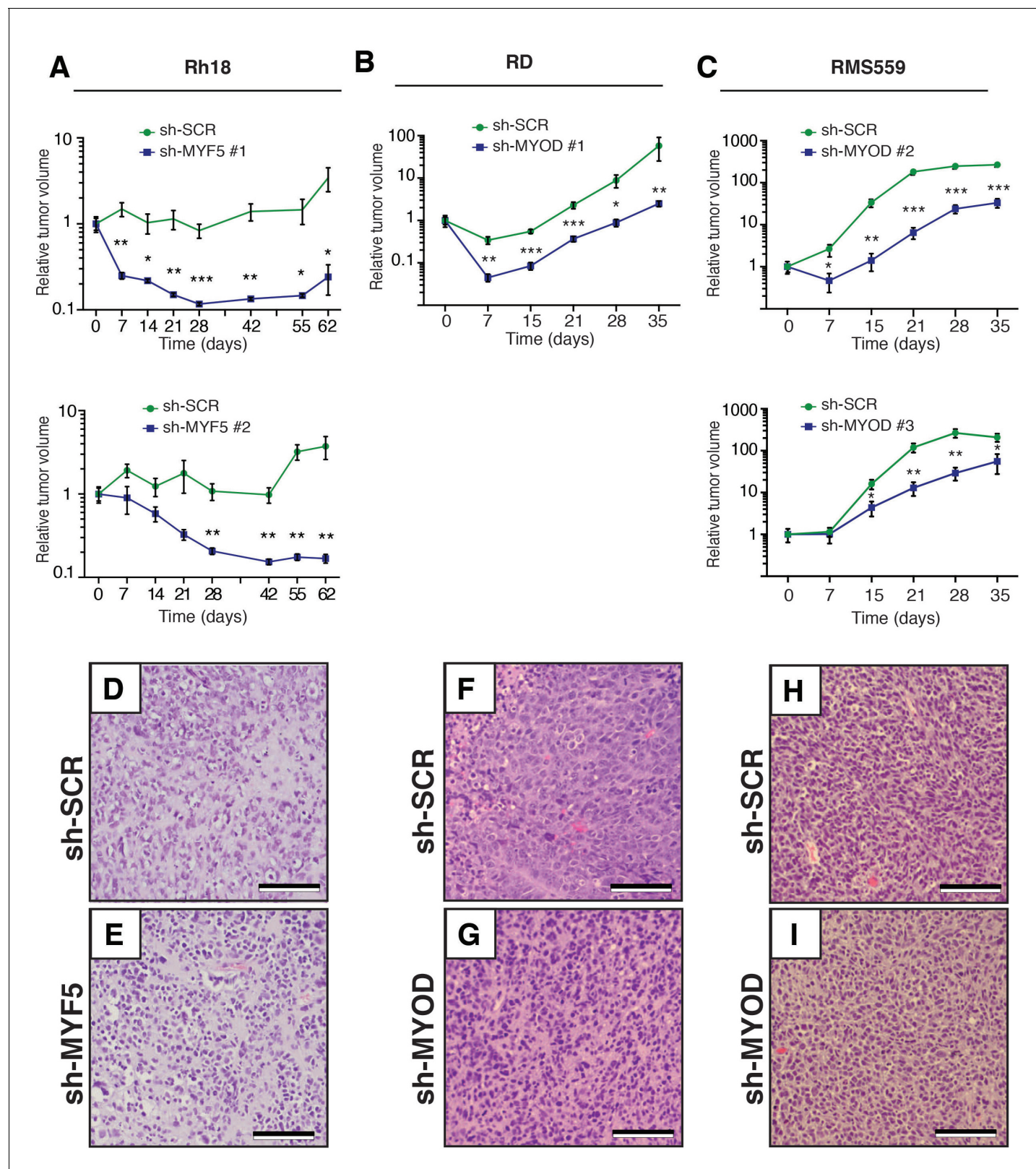


Figure 5—figure supplement 1. MYF5 and MYOD are required for human ERMS growth and maintenance following xenograft transplantation into NOD/SCID/IL2g null mice. (A–C) Quantification of tumor growth when assessed by luciferase bioluminescence imaging over time. Rh18 (A), RD (B), and RMS559 (C). N = 6 animals per analysis. Error bars denote Standard Error of the Mean (SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by Student's t-test. (D–I) Figure 5—figure supplement 1 continued on next page

Figure 5—figure supplement 1 continued

Hematoxylin Eosin stained sections of representative tumors isolated from mice engrafted with shRNA expressing Rh18 (D–E), RD (F–G), and RMS559 (H–I). Scale bars equal 50 μ m.

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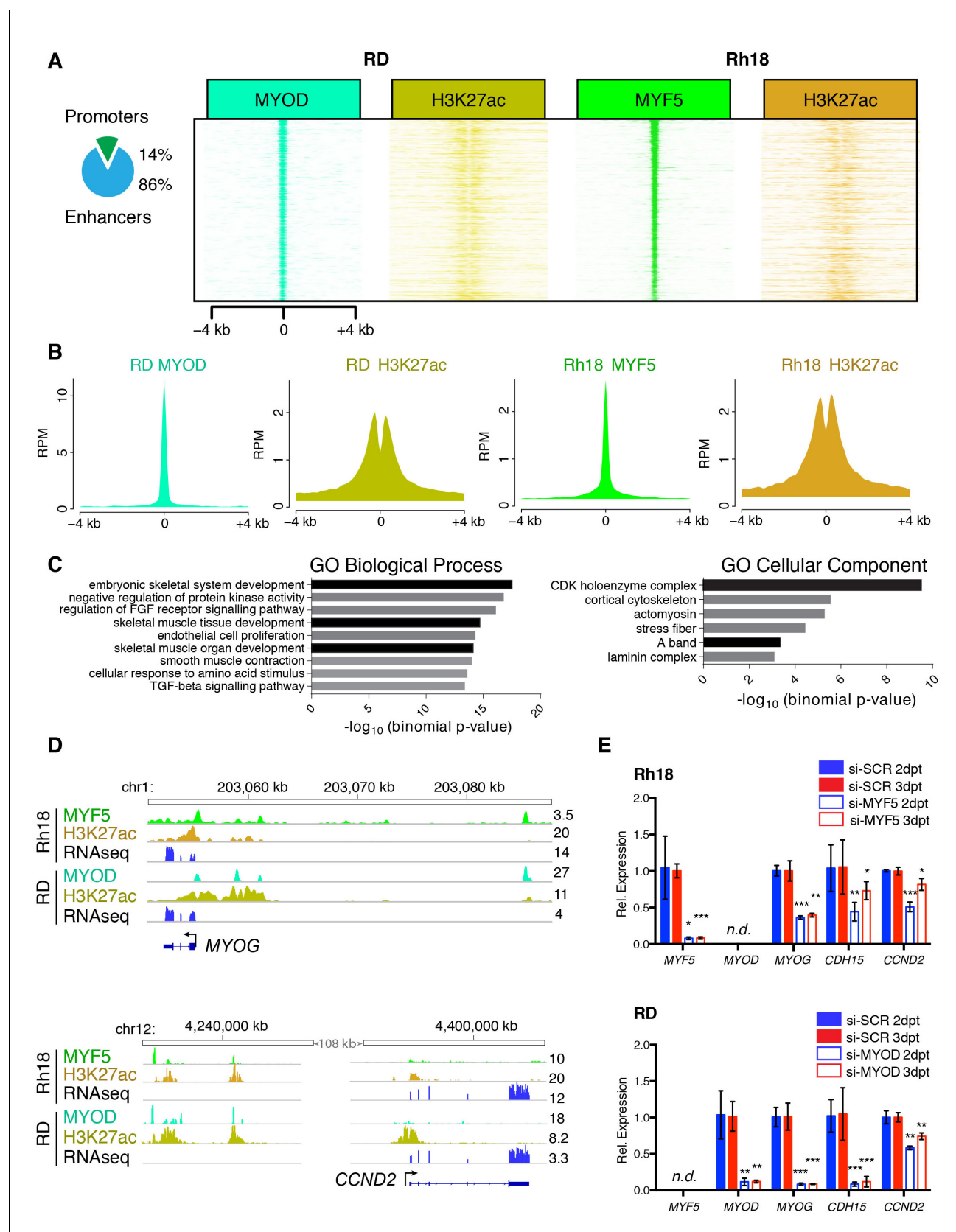


Figure 6. MYF5 and MYOD bind common promoter and enhancer regions and induce genes involved in muscle development and cell cycle. (A–B) ChIP-seq analysis showing genomic regions bound by both MYOD in RD cells and MYF5 in RH18 cells. H3K27 acetylation (H3K27ac). (C) Gene ontology Figure 6 continued on next page

Figure 6 continued

enrichment of gene regions bound by both MYOD in RD cells and MYF5 in RH18 cells. GO Biological Processes, GO Cellular Component predictions, and binomial p-values denoted. (D) Signal tracks for ChIP-seq and RNA-seq surrounding *MYOG* (top) and *CCND2* (bottom). Numbers to the right indicate reads per million mapped reads. (E) Quantitative real-time PCR gene expression analysis of RH18 (top) and RD cells (bottom). Cells were assessed following siRNA-mediated knockdown at 2 days (2dpt, blue bars) or 3 days post-transfection (three dpt, red bars). Error bars denote standard deviation. Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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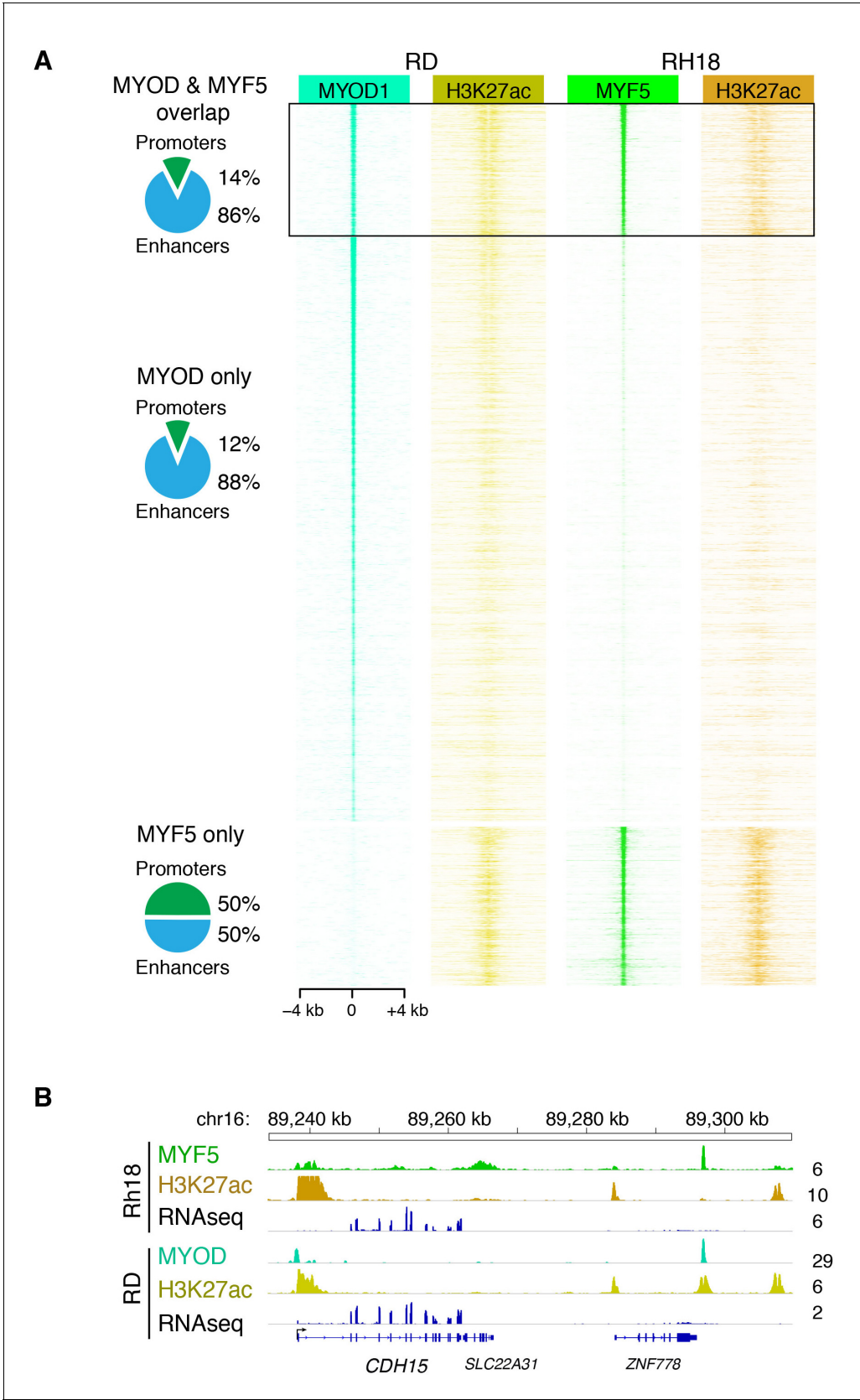


Figure 6—figure supplement 1. MYF5 and MYOD bind common promoter and enhancer regions. (A) ChIP-seq identified genomic locations bound by MYOD in RD cells, MYF5 in RH18 cells, and H3K27 acetylation (H3K27ac). Common binding sites are denoted by boxed region at the top and

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Figure 6—figure supplement 1 continued

reproduced in **Figure 6**. (B) Signal tracks for ChIP-seq and RNA-seq surrounding *CDH15*. Numbers to the right indicate reads per million mapped reads.

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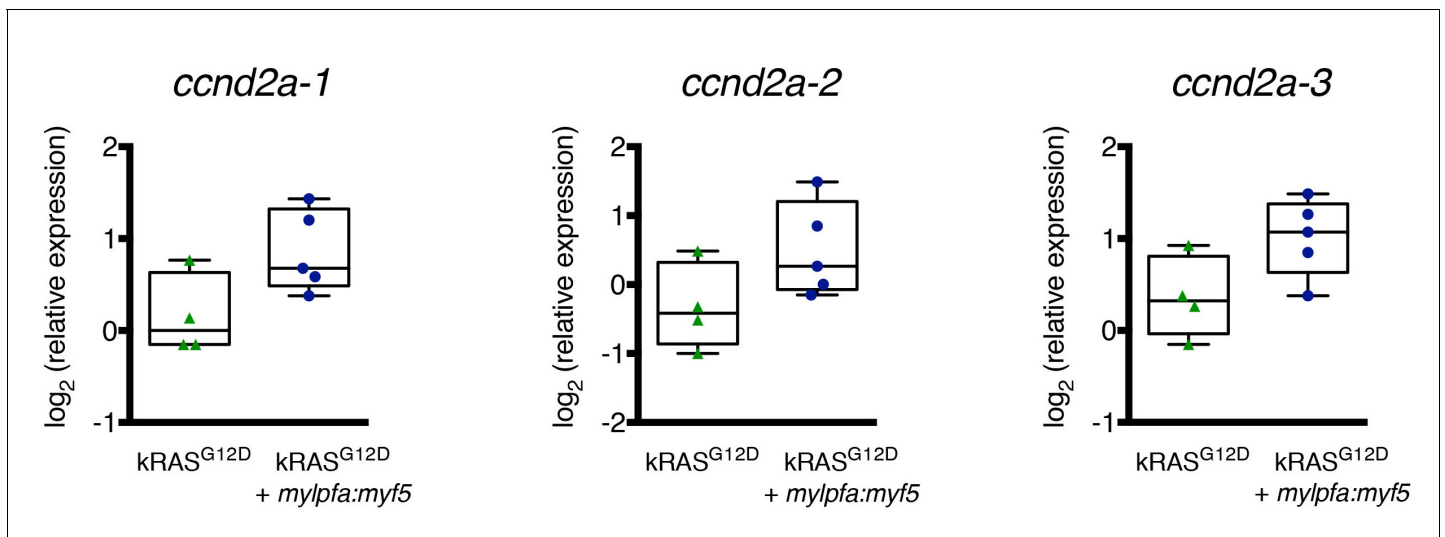


Figure 6—figure supplement 2. *Ccnd2a* expression in zebrafish ERMS. Quantitative real-time PCR gene expression performed on bulk zebrafish ERMS cells, comparing *ccnd2a* expression in zebrafish ERMS that express *kRAS*^{G12D} alone (K, N = 4) or co-express *mylpfa:myf5* (K+M, N = 5). Average gene expression with 50% confidence intervals denoted by box. Mean, maximum, and minimum also denoted. Three independent primer pairs confirm a trend toward higher *ccnd2a* expression in *mylpfa:myf5* expressing ERMS.

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