**PAX3-FOXO1 drives miR-486-5p and represses miR-221 contributing to the pathogenesis of alveolar rhabdomyosarcoma**

Jason A. Hanna, Matthew R. Garcia, Alicia Lardennois, Patrick J. Leavey III, Dino Maglic, Alexandre Fagnan, Jonathan C. Go, Jordan Roach, Yong-Dong Wang, David Finkelstein, and Mark E. Hatley

## Supplementary Information Inventory

## Supplementary materials and methods

*Immunocytochemistry*

Immunocytochemistry performed as previously described.[1] In brief, cells were grown on coverslips for 5 days in growth media or differentiation media (2% horse serum) with and without 50 ng/mL doxycycline. Cells were then fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in 15% normal goat serum (10000C, Invitrogen). Primary and secondary antibodies (Supplementary Table S5) were serially incubated for 1 hour at room temperature. VectaShield with DAPI (H-1500, Vector Laboratories, Burlingame, California, USA) was used for mounting the coverslips. Images captured on a Nikon Eclipse 80i at a magnification of 40x. The differentiation index was quantified by counting the number of MHC positive nuclei relative to the total number of nuclei in 4 random fields.

## Supplementary References


1 Hanna JA, Garcia MR, Go JC, Finkelstein D, Kodali K, Pagala V *et al*. PAX7 is a required target for microRNA-206-induced differentiation of fusion-negative rhabdomyosarcoma. Cell Death Dis 2016; 7: e2256.

2 Hinson AR, Jones R, Crose LE, Belyea BC, Barr FG, Linardic CM. Human rhabdomyosarcoma cell lines for rhabdomyosarcoma research: utility and pitfalls. Frontiers in oncology 2013; 3: 183.

## Supplementary tables

Supplementary Table S1. Candidate microRNAs regulated by PAX3-FOXO1 and differentially expressed in FN-RMS and FP-RMS.

| miRNA | log2 fold change FP-RMS vs FN-RMS | log2 fold change PAX3-FOXO1 shRNA | miRVana | Viability | Apoptosis |
|---|---|---|---|---|---|
| miR-221 | -3.61 | 0.42 | Mimic | Decreased | Increased |
| miR-18a | 0.91 | -0.23 | AntimiR | NC* | NC* |
| miR-301b | 0.96 | -0.6 | AntimiR | NC* | NC* |
| miR-301a | 1.14 | -0.35 | AntimiR | NC* | NC* |
| miR-510 | 1.25 | -1.57 | AntimiR | NC* | NC* |
| miR-506 | 1.29 | -1.66 | AntimiR | NC* | NC* |
| miR-19a | 1.32 | -0.32 | AntimiR | NC* | NC* |
| miR-335 | 1.61 | -0.23 | AntimiR | NC* | NC* |
| miR-326 | 1.62 | -0.61 | AntimiR | Decreased | NC* |
| miR-9 | 2.733 | -0.095 | AntimiR | NC* | NC* |
| miR-486 | 3.16 | -0.56 | AntimiR | Decreased | NC* |

*NC – no change

**Supplementary Table S2.** RMS cell line STR authentication.

| | Cell Line | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **LHCN** | **RD** | **Rh3** | **Rh2** | **Rh4** | **Rh18** | **Rh28*** | **Rh30** | **Rh41** |
| D3S1358 | 16, 18 | 15, 17 | 14, 17 | 15, 16 | 17 | 16 | 15, 16 | 15 | 17 |
| TH01 | 8, 9 | 9.3 | 7 | 7, 8 | 7, 9.3 | 7 | 9, 9.3 | 9, 9.3 | 7, 9.3 |
| D2S11 | 29, 30 | 28, 29 | 31.2, 32.2 | 29, 30 | 29, 31 | 32.2, 33.2 | 28, 30 | 29, 31.2 | 29, 31 |
| D18S51 | 12, 18 | 13, 18 | 15, 20 | 12, 16 | 15, 16 | 16, 17 | 14, 18 | 15, 16 | 15, 16 |
| Penta E | 11, 16 | 12 | 5, 15 | 13, 15 | 11, 17 | 8, 13 | 7, 12 | 7, 17 | 11, 17 |
| D5S818 | 11, 13 | 11 | 7, 11 | 10, 11 | 10, 13 | 12 | 11 | 12, 13 | 10, 13 |
| D13S317 | 11, 13 | 13 | 12, 13 | 12 | 8, 9 | 12 | 10 | 11 | 8, 9 |
| D7S820 | 9, 11 | 8, 12 | 10,11 | 12 | 10, 11 | 8, 10 | 8 | 10 | 10, 11 |
| D16S539 | 12 | 10, 11 | 11, 12 | 11, 12 | 12, 13 | 9, 12 | 8, 9 | 12 | 12, 13 |
| CSF1PO | 10, 11 | 10, 11 | 10, 12 | 12 | 11, 12 | 10 | 11 | 10, 11 | 11, 12 |
| Penta D | 10, 12 | 11, 13 | 14, 16 | 13 | 9, 12 | 7, 12 | 10, 12 | 11, 12 | 9, 12 |
| Amelogenin | XY | X | XY | X | X | X | XY | XY | X |
| vWA | 15, 17 | 18 | 14, 17 | 16, 17 | 16, 18 | 15, 17 | 16, 18 | 17, 18 | 16, 18 |
| D8S1179 | 10, 12 | 11, 15 | 12, 13 | 12,14 | 10, 13 | 13,15 | 11, 15 | 12, 15 | 10, 13 |
| TPOX | 8 | 9 | 8, 9 | 9, 11 | 8, 11 | 8, 9 | 8 | 8, 11 | 8, 11 |
| FGA | 20, 21 | 20,21 | 22 | 23, 24 | 20, 22 | 23 | 21, 23 | 22 | 20, 22 |

*Inconsistent with previously published STR, however matches STR of original xengraft.[2]

Supplementary Table S3. qRT-PCR primers and Taqman Probes (Applied Biosystems).

| **SYBR Primers** | | |
|---|---|---|
| **Gene** | **Primer 1** | **Primer 2** |
| *ALK* | TTTGTTGGTGATTCCAAGGAG | GCAGAGAGGGAAGGCTGTC |
| *ARID4B* | CATCAGTGCCCACTGTCAAA | GCAGCTGAACCTGGTGTTTT |
| *CCND2* | ACGGTACTGCTGCAGGCTAT | AGCTGCTGGCTAAGATCACC |
| *CDK6* | TGTCTGTTCGTGACACTGTGC | ATGCCGCTCTCCACCAT |
| *CKM* | CTCCTTCTCCGTCATGCTCT | GGTGGAGAAGCTCTCTGTGG |
| *CYCLD* | CTCCTTTCCTGCGTCACACT | TTTGATGGAGTGCAGCTTTG |
| *EMP* | GAGTTCTGAAGGGTCCCAGC | TGCGGTCACATACTTCCAGA |
| *ERBB3* | TCACACTCAGGCCATTCAGA | GTGCTGGGCTTGCTTTTC |
| *FGFR4* | CCTCCAGGGACAAGACTGG | AGGAGCCAGGTGAGGAGG |
| *FOXO1* | GCACACGAATGAACTTGCTG | AAGAGCGTGCCCTACTTCAA |
| *GRIN2A* | GACGCTCCAAACTGGAAGAA | TATCTCCTCCCACACCTTCG |
| *KIT* | GATGGATGGATGGTGGAGAC | GGGATTTTCTCTGCGTTCTG |
| *MET* | TGTTCGATATTCATCACGGC | GCATTTTTACGGACCCAATC |
| *PAX3-FOXO1* | TCCAACCCCATGAACCCC | GCCATTTGGAAAACTGTGATCC |
| *PIK3AP1* | GGAAACAACCTTCTCGTCCTC | CTACGTGGCAGCTGTGAAAA |
| *PIK3R1* | TTGATAAGAAGAGGCGGGG | GGTTCTTCGAAAACTGAAGCA |
| *PNPLA4* | GGGGGAGATGGTTACTGTCC | GCAGGACTGAAGCTAGTGGAA |
| *PTEN* | CGGTGTCATAATGTCTTTCAGC | TGAAGGCGTATACAGGAACAAT |
| *SMARCA5* | TTGGAGGCAAACTCTTTTCAA | TACAAACAACTGCCTTGGGG |
| *SMARCD2* | CCCTGCAGTTCCTGCACTAT | GCCATCAAAAAGCCTCTGAC |
| *SOCS2* | GGAGGACGGATGACAAAGTC | AGACACTCTCCGGACTGAGG |
| *TOB1* | TTCACTGGTCCCTTTTCACC | ATTGATGATGTTCGTGGCAA |
| *TWF1* | ATCCCAGGAATCTGAAGGCT | TCTTTGCCAGAGCCAGAAAT |
| | | |
| ***Taqman Probe*** | **Assay ID** | |
| *18S* | 4308329 | |
| *ARID1A* | Hs00195664_m1 | |
| *sANK1* | Hs00252830_m1 | |
| *U6* snRNA | 4427975-001973 | |
| hsa-miR-18a | 4427975-002422 | |
| hsa-miR-19a | 4427975-000395 | |
| hsa-miR-9 | 4427975-000583 | |
| hsa-mir-221 | 4427975-000524 | |
| hsa-miR-301b | 4427975-002392 | |
| hsa-miR-301a | 4427975-000528 | |
| has-miR-510 | 4427975-002241 | |
| hsa-miR-506 | 4427975-001050 | |
| hsa-miR-326 | 4427975-000542 | |
| hsa-miR-486 | 4427975-001278 | |
| hsa-miR-335 | 4427975-000546 | |

Supplementary Table S4. PCR Primers and oligonucleotides used for cloning.

| | |
|---|---|
| **PAX3-FOXO1 shRNA** | |
| Target Sequence | TCTCACCTCAGAATTCAATTC |
| Fwd Oligo (5'-3') | CCGGTCTCACCTCAGAATTCAATTCCTCGAGGAATTGAATTCTGAGGTGAGATTTTTG |
| Rev Oligo (5'-3') | AATTCAAAAATCTCACCTCAGAATTCAATTCCTCGAGGAATTGAATTCTGAGGTGAGA |
| **Scrambled shRNA** | |
| Target Sequence | CCTAAGGTTAAGTCGCC |
| Fwd Oligo (5'-3') | CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG |
| Rev Oligo (5'-3') | AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG |
| **PAX3-FOXO1 DsiRNA** | |
| Target Sequence | TCTCACCTCAGAATTCAATTC |
| Antisense Oligo (5'-3') | rUrArU rGrArC rGrArA rUrUrG rArArU rUrCrU rGrArG rGrUrG rArGrA |
| **Molecular Cloning Primers for pBabe-PAX3-FOXO1-Puro** | |
| PAX3-FOXO1 Fwd | CCGGATCCGCGAAGTGCCCCCAGG |
| PAX3-FOXO1 Rev | CCGTCGACCCT GCTCACTAACCCTCAGCC |
| **psiCHECK2 Sensor Oligos** | |
| miR-221 sensor Fwd | TCGAGGAAACCCAGCAGACAATGTAGCTGC |
| miR-221 Sensor Rev | GGCCGCAGCTACATTGTCTGCTGGGTTTCC |
| miR-486 Sensor Fwd | TCGAGATCCTGTACTGAGCTGCCCCGGTCGACATCCTGTACTGAGCTGCCCCGGC |
| miR-486 Sensor Rev | GGCCGCCGGGGCAGCTCAGTACAGGATGTCGACCGGGGCAGCTCAGTACAGGATC |
| **psiCHECK2 Site Directed Mutagenesis Primers of 3'UTRs** | |
| *CCND2* Primer 1 | AGTTTTGGTTATGGCCCATATAGACATTTCCCCATTAGATAGCAATATGGTGG |
| *CCND2* Primer 2 | CCACCATATTGCTATCTAATGGGGAAATGTCTATATGGGCCATAACCAAAACT |
| *TWF1* Primer 1 | TACTTTATATCAACATGGAATGATTTCAGTTCTAAGGTACTAAAAGCTGGACTTTTAAAAAACTAGTAT |
| *TWF1* Primer 2 | ATACTAGTTTTTTAAAAGTCCAGCTTTTAGTACCTTAGAACTGAAATCATTCCATGTTGATATAAAGTA |
| **Molecular Cloning Primers for pCMV6 Expression Constructs** | |
| pCMV6-miR-486 Fwd | CCCCGAATTCGCAGTGTGGCCACAGAGCA |
| pCMV6-miR-486 Rev | CCCCGTCGACCAGAAAGCTAGAGCCTCCTTGC |
| pCMV6-MYOD1 Fwd | AAGCTTGAATTCCTTTGCTATCTACAGCTTGGGTTGGG |
| pCMV6-MYOD1 Rev | GGATCCGTCGACTCAGAGCACCTGTGATATCGGGTTG |
| pCMV6-PAX3-FOXO1 Fwd | CCGTCGACGCGAAGTGCCCCCAGG |
| pCMV6-PAX3-FOXO1 Rev | CCGGATCCCCTGCTCACTAACCCTCAGCC |

Supplementary Table S5. Mutations of miRNA recognition sites in target gene 3'UTRs.

| Gene | Location | Original Site* | Mutated Site** |
|---|---|---|---|
| *CCND2* miR-221 Site | 761-767 | CUAUCUAAUGGGGAAAUGUAGCU | CUAUCUAAUGGGGAAAUGUCUAU |
| *CDK6* miR-221 Site A | 6813-6820 | UAAACAAAUUAUCUCAUGUAGCA | UAAACAAAUUAUCUCAUGUGAUA |
| *CDK6* miR-221 Site B | 6968-6974 | GCCAAGAACUAUGACUGUAGCAC | GCCAAGAACUAUGACUGUGAUAC |
| *ERBB3* miR-221 Site | 175-181 | CAAAAUUCUUAUGGUAUGUAGCC | CAAAAUUCUUAUGGUAUGUGAUC |
| *TWF1* miR-486 Site | 52-59 | AAAGUCCAGCUUUUAGUACAGGA | AAAGUCCAGCUUUUAGUACCUUA |

*microRNA recognition site underlined

**nucleotides mutated highlighted in red.

Supplementary Table S6. Antibodies used for immunoblots, immunocytochemistry, and immunohistochemistry.

| Immunoblot antibodies | | | | |
|---|---|---|---|---|
| **Antibody** | **Supplier** | **Product No.** | **Dilution** | **Diluent** |
| AKT | Cell Signaling Technology | 9272 | 1:1000 | BSA |
| Phospho-AKT (Ser473) | Cell Signaling Technology | 4060 | 1:1000 | BSA |
| CDK6 | Cell Signaling Technology | 3136 | 1:1000 | Milk |
| CCND2 | Cell Signaling Technology | 3741 | 1:1000 | BSA |
| ERBB3 | Cell Signaling Technology | 12708 | 1:1000 | Milk |
| FOXO1 | Cell Signaling Technology | 2880 | 1:1000 | BSA |
| GAPDH | EMD Millipore | MAB374 | 1:10000 | Milk |
| PAX3 | Developmental Studies Hybridoma Bank | Supernatant | 1:500 | Milk |
| PTEN | Cell Signaling Technology | 9559 | 1:1000 | BSA |
| TWF1 | Cell Signaling Technology | 8461 | 1:1000 | BSA |
| Immunocytochemistry antibodies | | | | |
| **Antibody** | **Supplier** | **Product No.** | **Dilution** | |
| MHC | Developmental Studies Hybridoma Bank | MF 20 | 1:25 | |
| Alexa 488 goat anti-mouse | Invitrogen | A-11029 | 1:500 | |
| Alexa 488 goat anti-mouse | Invitrogen | A-11029 | 1:500 | |
| Immunohistochemistry antibodies | | | | |
| **Antibody** | **Supplier** | **Product No.** | **Dilution** | **Retrieval** |
| Ki67 | ThermoFisher Scientific | RM-9106 | 1:100 | HIER |
| Myogenin | Dako | M3559 | 1:200 | HIER |
| TurboGFP | ThermoFisher Scientific | PA5-22688 | 1:1000 | HIER |

Supplementary Table S7. *miRVana* mimics and antimiRs.

| miRNA | miRVana | ID | Catalog Number |
|---|---|---|---|
| miR-18a | AntimiR | MH12973 | 4464084 |
| miR-19a | AntimiR | MH10649 | 4464084 |
| miR-301a | AntimiR | MH10978 | 4464084 |
| miR-301b | AntimiR | MH12929 | 4464084 |
| miR-326 | AntimiR | MH10686 | 4464084 |
| miR-335 | AntimiR | MH10063 | 4464084 |
| miR-486-5p | AntimiR | MH10546 | 4464084 |
| miR-506 | AntimiR | MH10709 | 4464084 |
| miR-510 | AntimiR | MH12923 | 4464084 |
| miR-9 | AntimiR | MH10022 | 4464084 |
| Negative Control | AntimiR | 4464058 | 4464058 |
| miR-221-3p | Mimic | MC10337 | 4464084 |
| Negative Control | Mimic | 4464058 | 4464058 |

**Supplementary Figure Legends**

**Supplementary Figure S1. PAX3-FOXO1 knockdown in Rh30 cells decreases proliferation, migration, and invasion. (a)** Immunoblot analysis of stably transduced Rh30 Tet-pLKO cells treated with doxycycline for 5 days. **(b)** immunoblot analysis in Rh30 cells as in (a) showing PAX3 expression is not altered with doxycycline treatment and knockdown of PAX3-FOXO1. **(c)** Expression of *PAX3-FOXO1* and PAX3-FOXO1 transcriptional targets by qRT-PCR in cells as in (a). **(d)** Population doubling is decreased with PAX3-FOXO1 knockdown. **(e)** Cell Titer Glo and **(f)** Caspase-Glo 3/7 Glo in cells treated with doxycycline for 3 days. **(g)** Low-density colony formation in Rh30 PAX3-FOXO1 knockdown cells and quantification of colonies. **P*<0.05, ***P*<0.01, ****P*<0.001 for shScr+Dox vs shP3F+Dox.

**Supplementary Figure S2. Knockdown of PAX3-FOXO1 induces myogenic differentiation. (a)** ICC for MHC (green) and DAPI (Blue) in Rh41 Tet-pLKO cells grown in growth media or differentiation media with and without 50ng/mL doxycycline treatment for 5 days. **(b)** Quantification of the differentiation index or percentage of MHC positive nuclei from four representative fields from (a). **(c)** Expression of *CKM* by qRT-PCR in cells as in (a). **(d)** ICC in Rh30 Tet-pLKO cells as in (a) and **(e)** quantified as differentiation index. **(f)** *CKM* expression by qRT-PCR in cells as in (c). Scale bars = 25 μm. **P*<0.05, ***P*<0.01, ****P*<0.001 for shScr+Dox vs shP3F+Dox.

**Supplementary Figure S3. MicroRNAs regulated by PAX3-FOXO1. (a)** Immunoblot analysis in Rh30, Rh41, and LHCN myoblasts and RD cells stably transduced with Empty Control vector or pBabe-PAX3-FOXO1 forced expression. **(b)** Expression of

known transcriptional targets of PAX3-FOXO1 by qRT-PCR in control and PAX3-FOXO1 overexpressing LHCN and RD cells as in (a). **(c)** Knockdown of PAX3-FOXO1 in Rh30 and Rh41 cells 5 days after transfection with Scrambled (Scr) or PAX3-FOXO1 siRNA as assessed by qRT-PCR for *PAX3-FOXO1*, *ALK*, and *MET*. **(d)** Immunoblot analysis of knockdown of PAX3-FOXO1 in cells as in (c). **(e)** Expression of candidate microRNAs after PAX3-FOXO1 knockdown by siRNA. **P*<0.05, ***P*<0.01, ****P*<0.001 for empty vs PAX3-FOXO1 or Scr siRNA vs P3F siRNA.

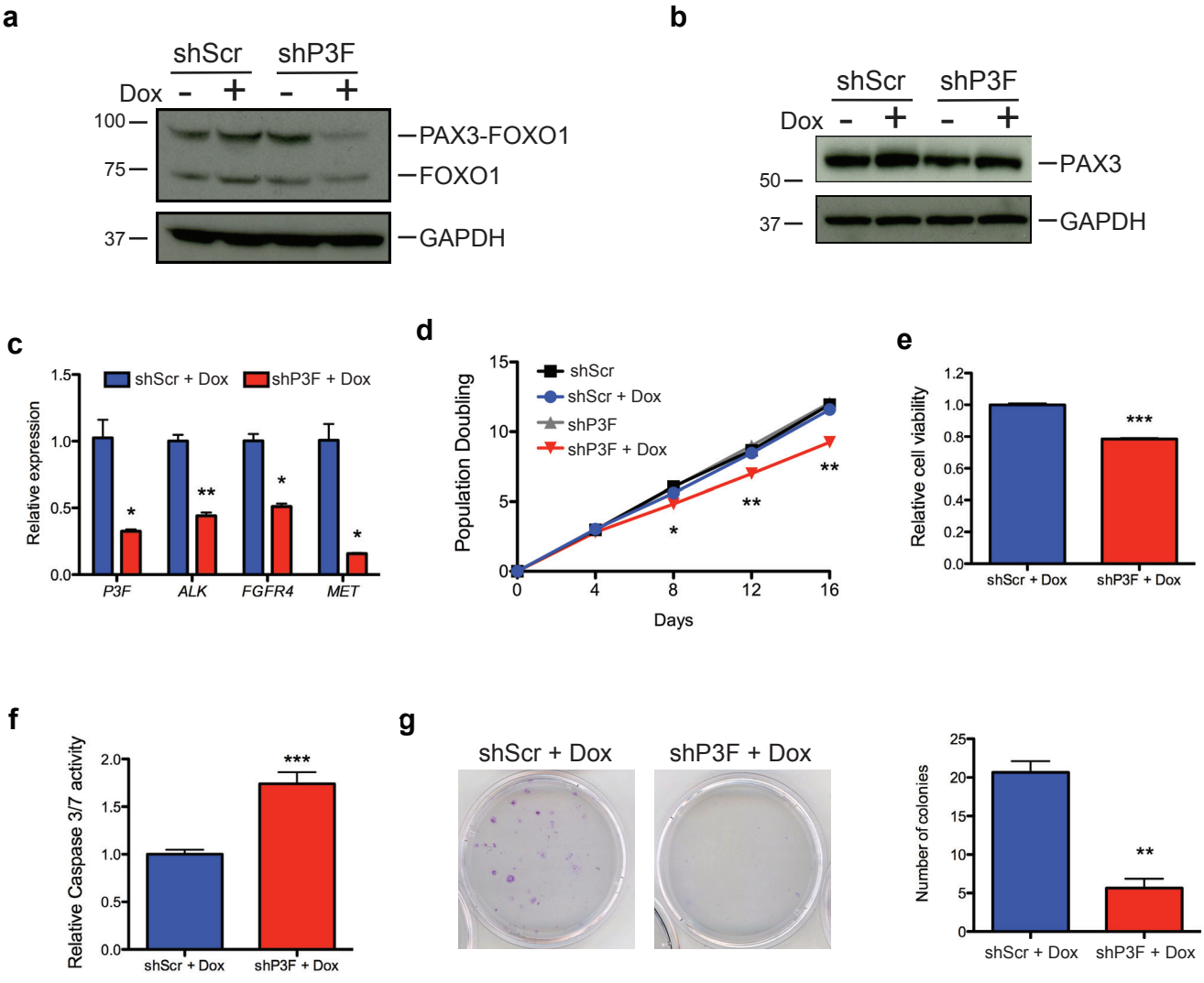
**Supplementary Figure S4. Target recognition sequences for miR-221-3p targets.** **(a)** miR-221 site in the 3'UTR of human *CCND2,* **(b)** *CDK6,* and **(c)** *ERBB3.*

**Supplementary Figure S5. Caspase 3/7 activity in antimiR transfected Rh41 cells.** Caspase 3/7 Glo activity in Rh41 cells transfected with NC (black) or indicated AntimiR (Red) 3 days after transfection.

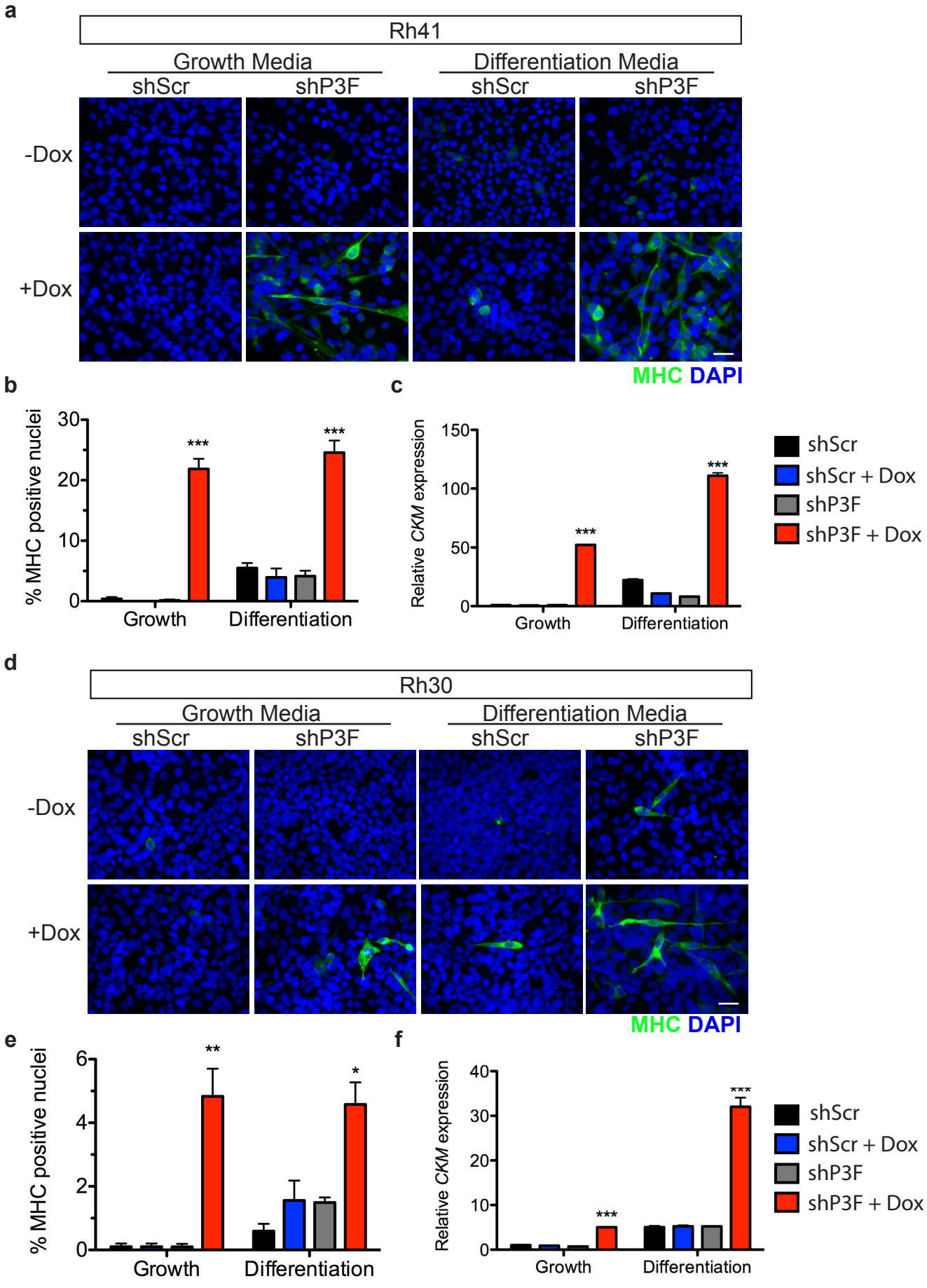
**Supplementary Figure S6. Oncogenic miR-486-5p target gene analysis in FP-RMS.** **(a)** Venn diagram of 2,755 putative miR-486-5p target genes and genes with increased expression with PAX3-FOXO1 knockdown. **(b)** Relative expression of putative miR-486-5p targets by qRT-PCR in Rh41 cells 72 hours after NC (black) or AntimiR-486-5p (red) transfection. **(c)** Immunoblot analysis in Rh30 and Rh41 whole cell lysates 72 hours after transfection with NC or AntimiR-486-5p. **(d)** Immunoblot analysis for TWF1 in cells as in (c). **(e)** miR-486-5p recognition site in the human *TWF1* 3'UTR . **(f)** Luciferase activity in C2C12 cells co-transfected with miR-486-5p or control vector and wild type or mutant miR-486 site TWF 3'UTRs reporters. Luciferase activity represented as mean (n=4) Renilla/Firefly luciferase activity ratio normalized to empty reporter (no miR-486-5p).

**Supplementary Figure S7. Inhibition of miR-486 in additional FP-RMS cells reduces cell viability. (a)** Phase contrast and GFP live cell fluorescent microscopy of indicated FP-RMS cells 48 hours following transduction with miRZip-Scr or miRZip-486-5p. The percentage of GFP positive cells indicating transfection efficiency lower left. Scale bar, 50μm. **(b)** qRT-PCR expression of miR-486-5p in cells from (a). **(c)** Cell Titer Glo cell viability assay in cells 7 days following transduction with miRZip-Scr or miRZip-486. **P*<0.05, ***P*<0.01, ****P*<0.001 for miRZip-Scr vs miRZip-486-5p comparisons.

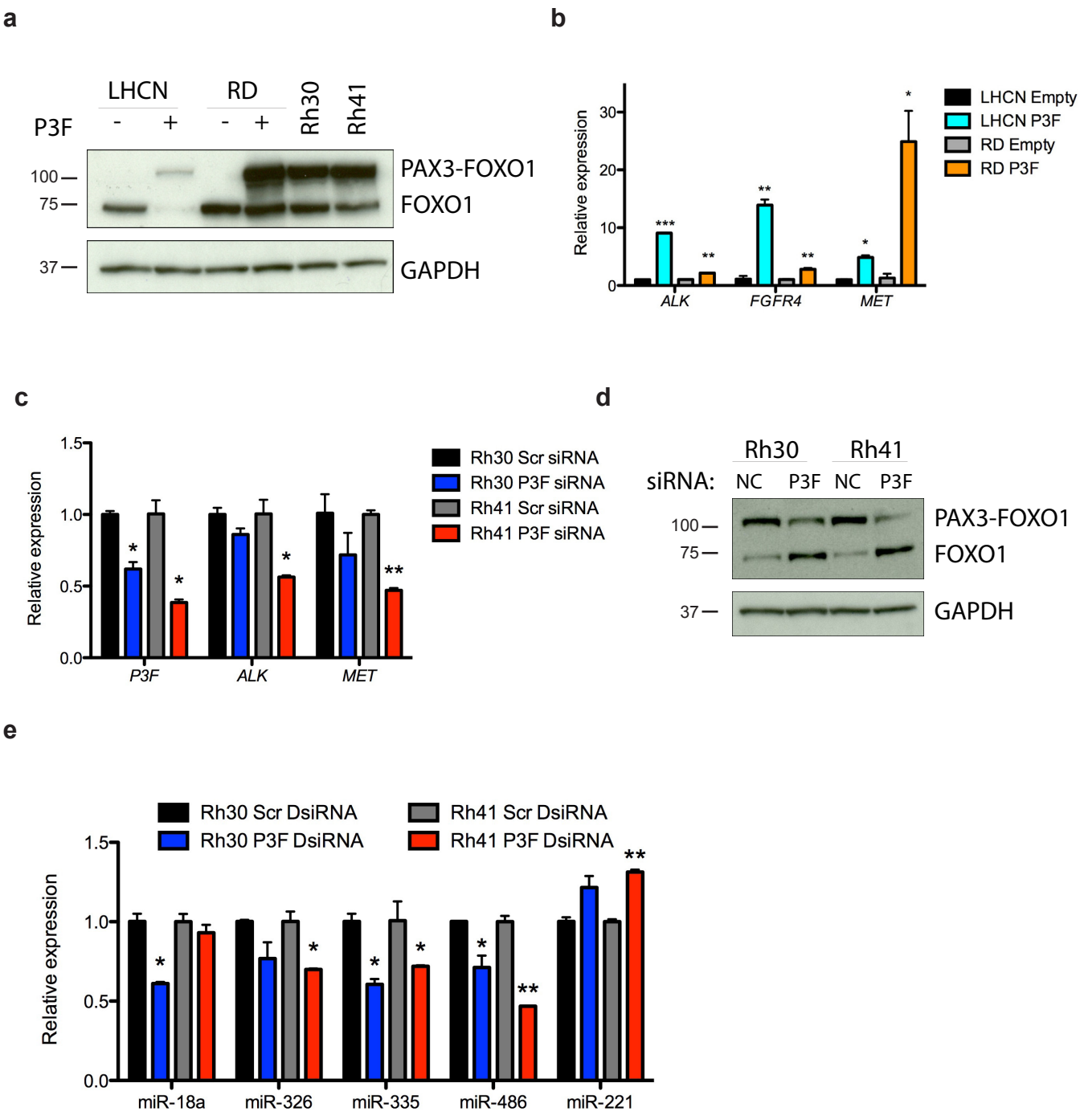
**Supplemental Figure S8. Xenograft growth is reduced with miRZip-486-5p. (a)** Final tumors and **(b)** tumor weights after xenograft growth in Rh30 and Rh41 cells transduced with miRZip-Scr or miRZip-486-5p as indicated. **(c)** qRT-PCR expression of miR-486 in xenografted Rh41 cells transduced with miRZip-Scr or miRZip-485-5p (p=0.82). **(d)** Whole-mount brightfield and GFP imaging of Rh41 xenografts. Scale bar, 4 mm. **(e)** H&E staining and IHC for MYOGENIN, Ki67, GFP/DAPI in Rh41 xenografts. Scale bar, 50 μm.



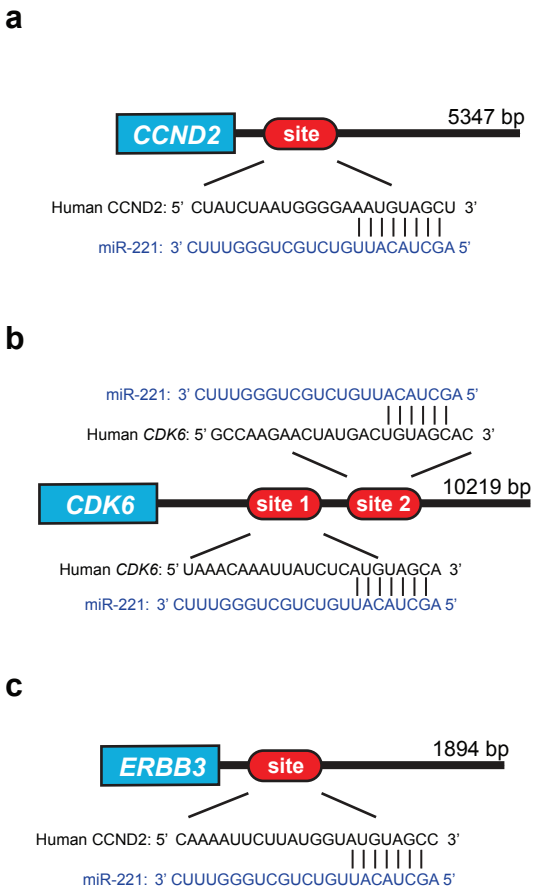

Hanna et al., Supplementary Figure S1.



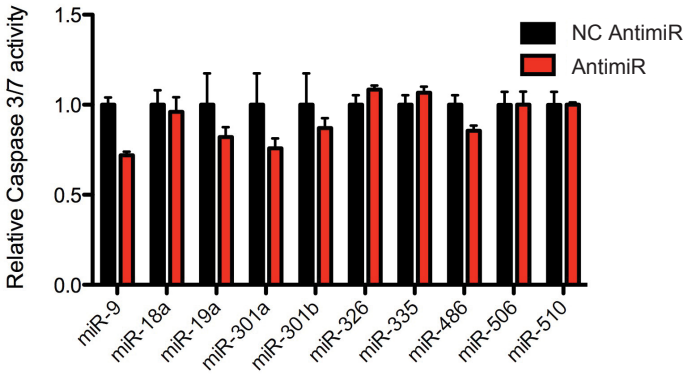

Hanna et al., Supplementary Figure S2.



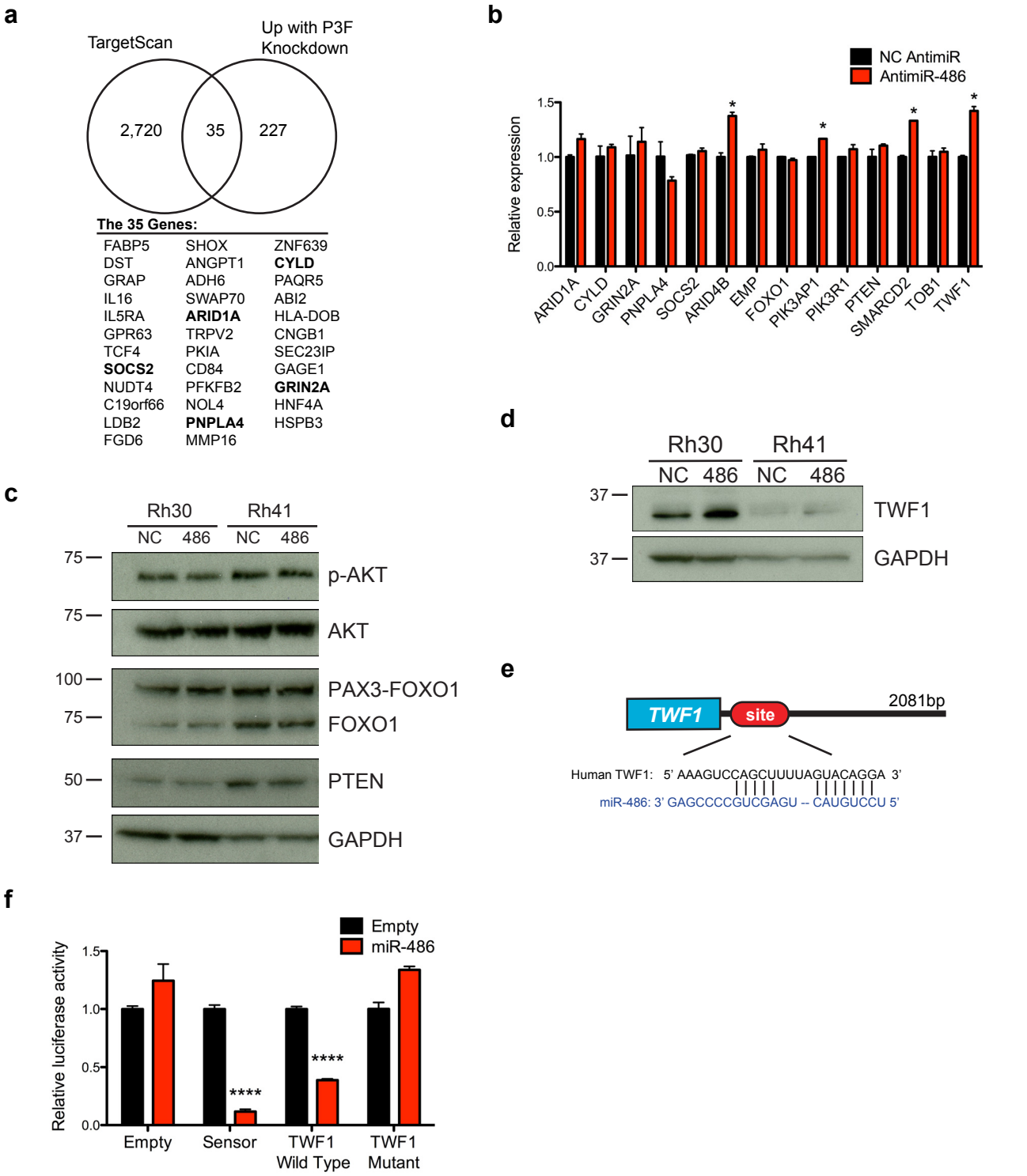

Hanna et al., Supplementary Figure S3.




Hanna et al. Supplementary Figure S4

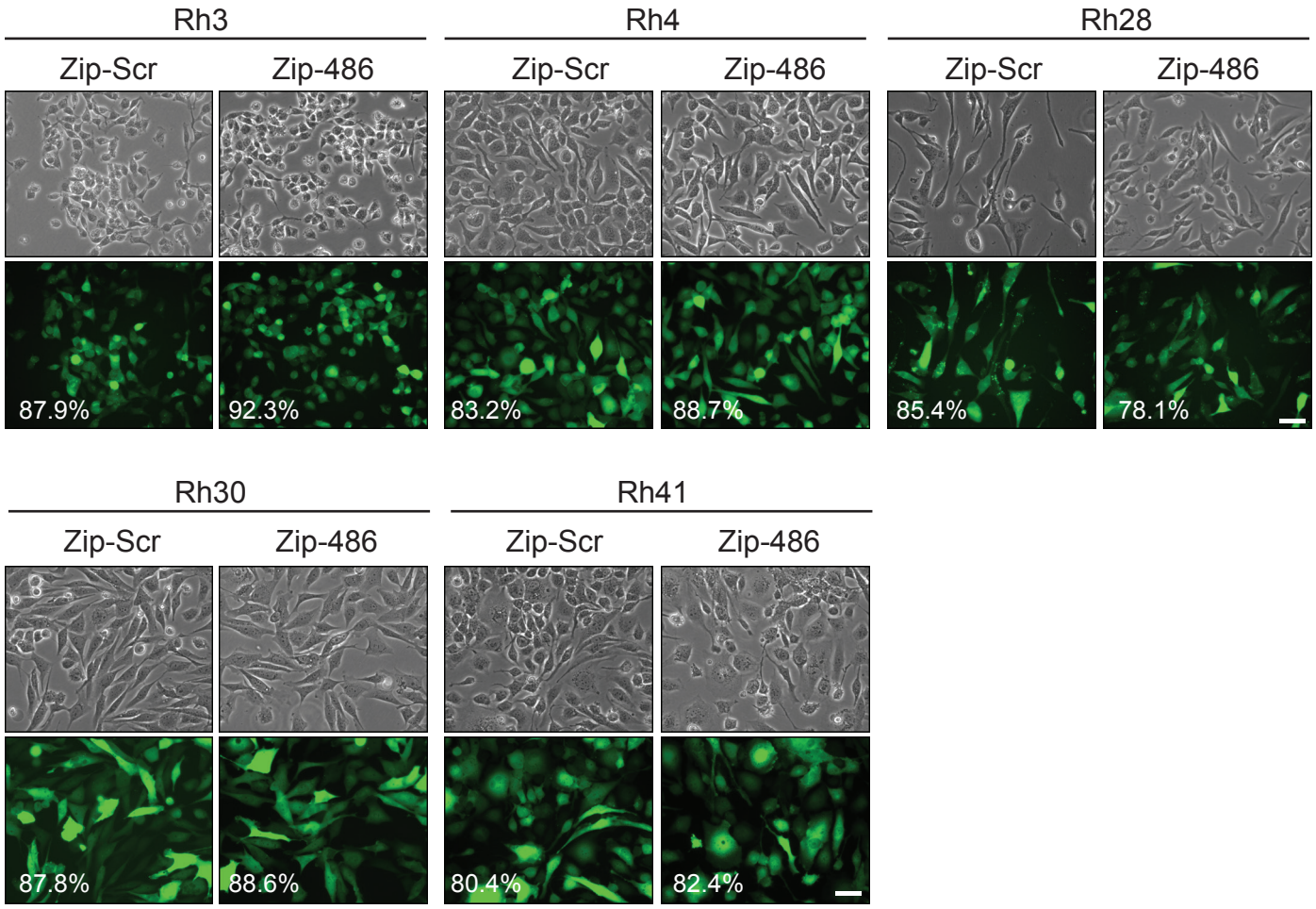



Hanna et al., Supplementary Figure S5.




Hanna et al. Supplementary Figure S6

a

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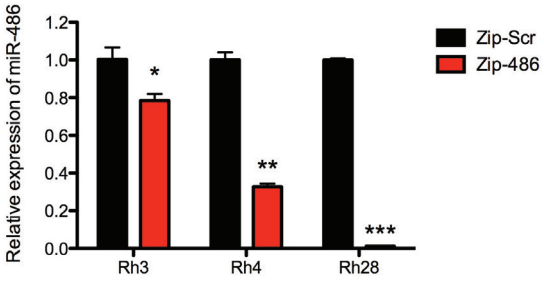


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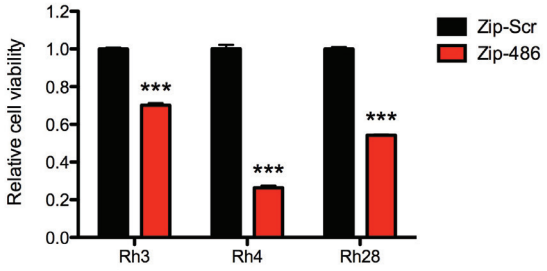
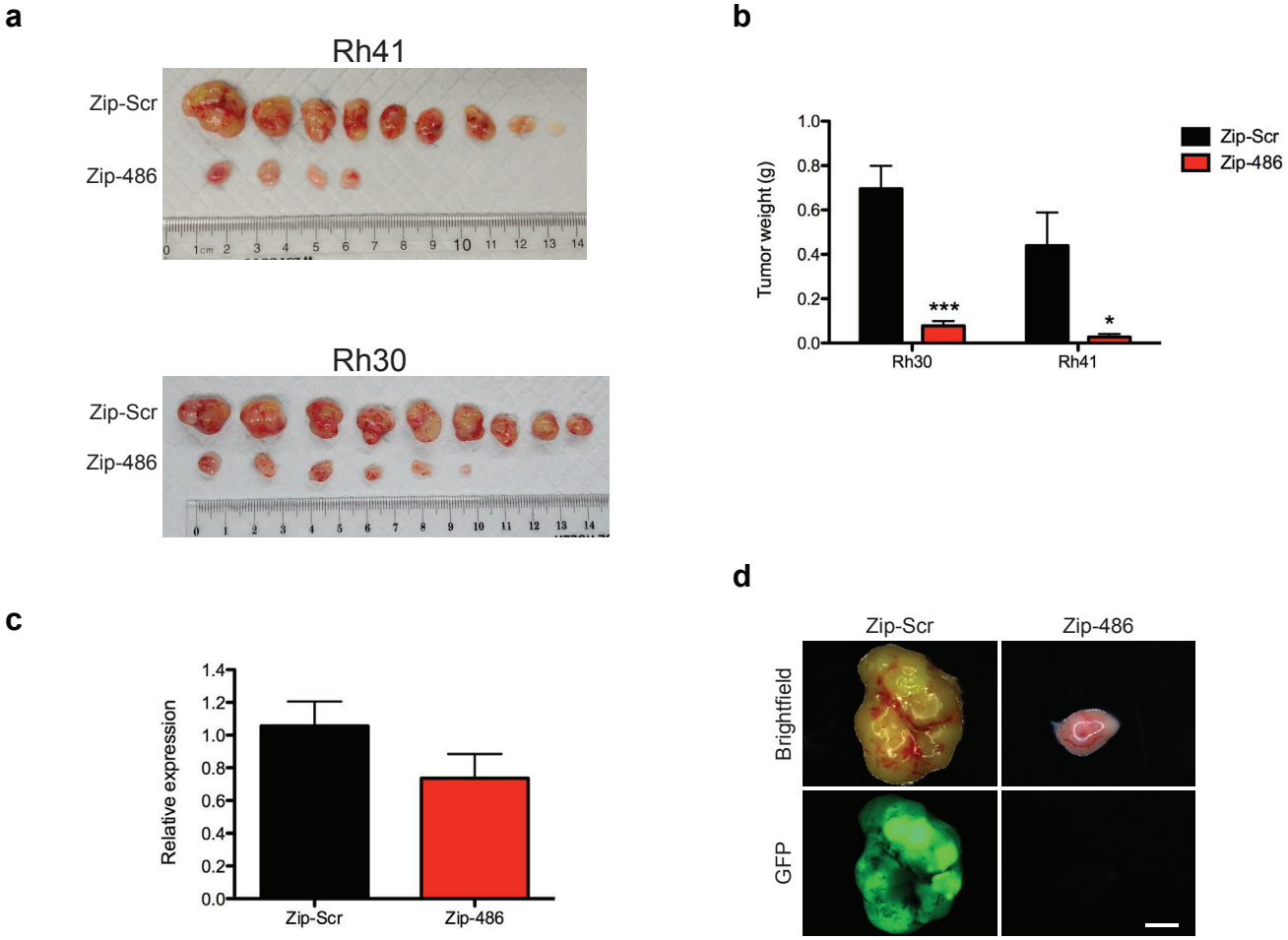



Hanna et al. Supplemental Figure S7.




Hanna et al. Supplemental Figure S8.