

## Supplemental Figure and Table Legends

**Supplemental Figure 1.** Characterization of pediatric mesenchymal progenitor cell isolates (pMPCs). A. FACS analysis on pMPCs for mesenchymal stem/progenitor surface markers: CD146, CD105, CD90, CD45 (blue) and control antibodies (red). B. Osteogenic differentiation of uninduced and induced pMPCs demonstrated using Alizarin Red S staining. C. Adipogenic differentiation of uninduced and induced pMPCs demonstrated using Oil Red O staining. D. Colony forming assay of pMPCs plated at low density, 100 cells/10cm plate and stained with Giemsa. (CFU-F = colony forming units-fibroblast like) E. Gene expression profiling on pMPCs show pMPCs clustered with Ewing sarcoma cells stably expressing an shRNA against EWS-FLI1.

**Supplemental Figure 2.** Expression of EWS-FLI1 in pMPCs results in gene expression changes relevant to Ewing sarcoma. A. Individual plots of the read counts for the 3 lines of pMPCs sequenced using the 3SEQ RNAseq method. Red represents upregulated targets and green represents downregulated targets. B. Heatmap of differentially expressed genes in the 3 pMPCs expressing EWS-FLI1 compared to uninfected pMPCs. (EF = EWS-FLI1, UI = uninfected) C-G. GSEA analysis showing enrichment of published lists of genes in our 3SEQ dataset. C. Geneset is genes upregulated by EWS-FLI1 in adult human mesenchymal stem cells (hMSCs). D. Geneset is genes upregulated by EWS-FLI1 in adult pediatric hMSCs. E. Geneset is genes induced by EWS-FLI1 expression in primary pediatric hMSCs grown in KnockOut (KO) serum. F. Geneset is genes induced by EWS-FLI1 expression in primary pediatric hMSCs that express CD133, thought to be a cancer stem cell like population of cells. G. Geneset is EWS-FLI1 induced targets identified by knockdown of EWS-FLI1 in a Ewing sarcoma cell line.

**Supplemental Figure 3.** Inc277 Transcript Analysis. A. RNAseq reads on the 3' end of Inc277 in each of 3 pMPCs isolates expressing EWS-FLI1. B. Chromosome 15 locus near Inc277. C. Location of northern probes (NP), northern probe primers, overexpression cloning primers, qPCR primers and shRNA seed sequences mapped onto the Inc277 locus. D. Northern blot for Inc277 using northern probe 1 (primers 1for/1rev). RPLPO and ethidium bromide (EtBr) staining of 28S and 18S RNAs used as loading controls.(Note this is the whole blot partially shown in Figure 3D of the main text). E. Northern blot for Inc277 using 3 other probes spanning largest isoform: Northern Probe 2 (primers 2for/2rev), Northern probe 3 (primers 3for/3rev) and Northern probe 4 (primers 4for/4rev). Ethidium bromide used to stain 28S and 18S for loading controls. F. RT-PCR analysis for EWS-FLI1 and Inc277 in two Ewing sarcoma cell lines with knock-down of EWS-FLI1 or GFP as a control. PCR primers for Inc277-2 are 1for/1rev and 2for/2rev. PCR primers for the isoform with the alternative 3' start site (LNC277-1) are 3for/2rev. Error bars are standard deviation for n=2.

**Supplemental Figure 4.** The long-non-coding RNA Inc277 is required for cell proliferation in Ewing tumor cells and is localized to both the nucleus and the cytoplasm. A. Cell proliferation assay in Ewing cell lines transduced with indicated shRNAs. Data normalized to uninfected cells. Error bars represent standard deviation for n=2-4. t-test was performed comparing colony numbers for shGFP vs. colony numbers for all three shRNAs together. \* p-value is <0.05, \*\* p-value < 0.01, \*\*\* p-value is <0.001 by two-tailed t-test. B. Inc277 depletion has no effect on cell proliferation of IMR90 cells. C. Immunofluorescence with anti-BrDU antibody in the Ewing cell line A673 infected with indicated hairpins. D. Quantitation of BrDU staining. E. RT-PCR for Inc277 in fractionated and total RNA isolated from Ewing cell lines. Expression relative to cytoplasmic expression. F. RT-PCR for KCNQ10T1 in fractionated and total RNA isolate from Ewing cell lines, used as a control for a nuclear enriched long non-coding RNA. Expression relative to cytoplasmic expression.

**Supplemental Figure 5.** Validation of shRNA knockdown for samples used for paired-end RNAseq. A. Schematic representation of paired-end RNAseq analysis. B. RT-PCR for EWS-FLI1 expression in A673 samples shGFP1, shGFP2, shEF1 and shEF2. RT-PCR normalized to shGFP1. (shEF = shEWS-FLI1) C. RT-PCR for lnc277 expression in A673 samples shGFP1, shGFP2 and shlnc1-shlnc5. RT-PCR normalized to shGFP1. (shlnc = shlnc277) D. Northern blot analysis showing the levels of lnc277 A673 samples shGFP1, shGFP2, shEF1, shEF2, shlnc2-shlnc5. E. RT-PCR for EWS-FLI1 in A673 samples shGFP3, shGFP4, shEF3 and shEF4. RT-PCR normalized to shGFP3. F. RT-PCR for lnc277 expression in A673 samples shGFP3, shGFP4, shlnc6 and shlnc7. RT-PCR normalized to shGFP3.

**Supplemental Figure 6.** lnc277 regulates a subset of genes downstream of EWS-FLI1. A. Principal component analysis of the RNAseq samples. Red = shGFP, Green = shEF and Blue = shlnc277. B. Scatter plots comparing shGFP versus shEF and shGFP versus shlnc277. Blue = overlap repressed targets between shEF and shlnc277 samples. C. RT-PCR of genes repressed by EWS-FLI1 or lnc277 that validated using 2 of 3 shRNAs targeting lnc277. RT-PCR normalized to shGFP. D. RT-PCR of genes induced by EWS-FLI1 or lnc277 that validated with at least 2 of the 3 shRNAs targeting lnc277. RT-PCR normalized to shGFP. E. DAVID analysis of repressed genes that overlap between EWS-FLI1 and lnc277.

**Supplemental Figure 7.** lnc277 overexpression does not consistently alter the growth and differentiation of pMPC cells. A. RT-PCR showing expression of lnc277 in pMPC cells. lnc277 expression is relative to GAPDH. # = not expressed B. Cell growth of pMPCs expressing lnc277. C. CFU-F formation of pMPCs expressing lnc277. D. Osteogenic differentiation of pMPCs expressing lnc277. E. Adipogenic differentiation of pMPCs expressing lnc277.

**Supplemental Table 1.** Microarrays used for generating the heatmap in Supplemental Figure 1E.

**Supplemental Table 2.** EWS-FLI1 target genes identified in pMPCs. Induced tab refers to the genes that are significantly upregulated in pMPCs expressing EWS-FLI1 compared to uninfected pMPCs. Repressed tab refers to the genes that are significantly downregulated in pMPCs expressing EWS-FLI1 compared to uninfected pMPCs. EF = EWS-FLI1 pMPCs, UI = uninfected pMPCs.

**Supplemental Table 3:** Paired end RNAseq results comparing shGFP to shEWS-FLI1 samples. Up and down regulated genes listed in separate sheets.

**Supplemental Table 4:** Paired end RNAseq results comparing shGFP to shInc277 samples. Up and down regulated genes listed in separate sheets.

**Supplemental Table 5:** Overlapping genes induced by both EWS-FLI1 and *Inc277* (decreased upon loss of EWS-FLI1 and *Inc277*, 61 genes) or repressed by both EWS-FLI1 and *Inc277* (induced upon loss of EWS-FLI1 and *Inc277*, 250 genes) based on an FDR <0.2 and a fold change cutoff of 2.0.

**Supplemental Table 6.** Pearson correlation for *Inc277* expression compared to each mRNA for the primary Ewing tumor samples described in Crompton et al. Third column indicates whether a gene was identified as repressed by *Inc277* in the A673 dataset (Table 5).

**Supplemental Table 7:** The annotation clusters identified using DAVID (The Database for Annotation, Visualization and Integrated Discovery) based on the shEWS-FLI1 and shInc277 overlapping repressed genes based on an FDR <0.2 and a fold change cutoff of 2.0.

**Supplemental Table 8:** List of *Inc277* interacting proteins from performing two human protein arrays with in vitro transcribed *Inc277*.

**Supplemental Table 9.** Paired end RNAseq results comparing shGFP to hnRNPK samples.  
Up and down regulated genes listed in separate sheets.

**Supplemental Table 10:** Description of the pMPC cell lines isolated from patients at Lucile Packard Children's Hospital, Stanford, CA.

## Supplemental Materials and Methods

### shRNA Target Sequences

shGFP

GCAAGCTGACCCTGAAGTTCAT

shFLI1

CGTCATGTTCTGGTTTGAGAT

shInc277-1 (TRCN0000129827)

CTCTATTTCTTCCTGCAACAT

shInc277-2 (TRCN0000130126)

CCATCTTCAAACCAGAAGATC

shInc277-3

GCATCACTAAAGCTGAATATA

shHNRPK-1 (TRCN0000062455)

GTCTGGGACTGAAACACTGGC

shHNRPK-2 (TRCN0000062456)

CGCGACGGTCATCAAACATCA

shSTAU1-1 (Thermo, VLHS 202941)

CGAGTAAAGCCTAGAATCAAA

shSTAU1-2 (Thermo, VLHS 262825)

TCCAGAGTGCTCGATACTGTT

shScrambled

CCTCCACCCTCACTCTGCCAT

## RT-PCR Primers

Gene	Forward	Reverse
ABI3	GCAGGAGACCCCTCAACTT	ACAGCTGCGTGCTGAGGT
ADCY4	ACCTGAGCCACGGAGACA	ACTGGGTGCTGAAGGAAGC
ALX4	ACAGCTGGCCATGAGGAC	GCTGCATCTGCCCAAAC
AXL	CACCCAGAGGTGCTAATGG	TCAACTCCTGCCTTCTCGTG
BMF	GAGACTCTCTCCTGGAGTCACC	CTGGTTGGAACACATCATCCT
CAV1	ACAAGCCCAACAACAAGG	ATCGGGATGCCAAAGAGG
CCDC155	AAGGAGCCATCCATGTGGT	CAGGGAGATCAGCCGTGA
CYP4F22	GACAGTGAACCCAAAAGAATTGA	GAACGCCGTCTTCTCCAG
DAPK2	ACGTGGTGCTCATCCTTGA	TGGCCTCCTCCTCACTCA
DHH	GCAACAAGTATGGGTTGCTG	CGGACCGCCAGTGAGTTA
ECHDC2	CAGGTGCAGACCTGAAGGAG	GCCGAGGAAGCGATGTCATT
EFNA1	TCCACAGGAGAAGAGACTTGC	AGTCCAGGCAAGTGGGAAG
EOMES	GTGGGGAGGTCGAGGTTC	TGTTCTGGAGGTCCATGGTAG
EWS-FLI1	GATCCTACAGCCAAGCTCCA	ATTGCCCAAGCTCCTCTTC
FATE1	CTGCTTCAGCCAAACGAGT	GGCCTGATTCTCTCAGCATT
FEV	CTTGTCTCCCCACCACTCC	AGTAGTGATATTGAATGGGGCTTC
FGF18	ATGAACCGCAAAGGCAAG	GAACACACACTCCTTGCTGGT
FMNL1	CTACGCGCCATCATGAACT	ACACAGGCTGGGTGGTTC
FOXE1	AACCTAAAGTCCCAGGATTGG	CGTCTGCTCAAAGTTCAA
GAPDH	CTCTGCTCCTCCTGTTGAC	ACGACCAAATCCGTTGACTC
GIMAP8	ACGGAGGACCCTATCATGTG	TGCAGCTTCATTACACAATC
GPR123	CACCTACATCGTGCACCAGA	GGCGAACACAGTGAAGGTC
GRASP	CCGAAAGGGCTCAGGATT	TCCTTCTCCAACGTCAGCA
HIST2H3C	CAAGCGCGTGACCATTATGC	CCACTTCTTAAGCCCGCTCT
HNRNPK	CCACTTGTTGCGGCCTAT	ACGGGCACACCAATCAGTTA
HPRT	TGACACTGGTAAAACAATGCA	GGTCCTTTTACCAGCAAGCT
ID2	GCCCAGCATCCCCAGAA	GGTGGTCAGCGGCGTCTCCT
IER3	CCCTCGAGTGGTCCGGC	CACCCTCTTCAGCCATCAGG
IFIT1	AGAACGGCTGCCTAATTTACAG	GCTCCAGACTATCCTTGACCTG
IFITM1	CCTTTGCACTCCACTGTGC	ATCTAGGGGCAGGACCAAG
IGF1	GGTGGATGCTCTTCAGTTCGT	CCACGATGCCTGTCTGAGG
IL1A	GACTCAGGCTTAAGCTGCCA	TGGCCATCTTGACTTCTTTGC
IL8	ACCGGAAGGAACCATCTCAC	GGCAAAACTGCACCTTCACAC
ISM2	GGGTCTTCAAGGATTCTGTCA	CAGTGCTGCAGTTCCCACT
JPH3	ACGGGGCCAAATACGAAG	CACTGGCCCTGGTAGGTC
KAZALD1	AACCTCACTGTGGCACACC	TGCCCTGTCACATTCCAAG
LASS1	TCCTACCATCGGCTGCAT	TAGAGGCGGAACCAGAACC
LNC277	TCCAAGACGCGTTTCAGAG	CCACAAAGAGTTTACCAAGGTGT
LOX	GCCTGCTGACGTTTAGGTCT	GCCCTGTATGCTGTACTION
MAFB	AGGGAAGCTGCCAAGCTC	ATTTGACCATAAGACAAGGCTGT
NGFR	TCATCCCTGTCTATTGCTCCA	TGTTCTGCTTGCAGCTGTTC
NKX2.2	GAACCCCTTCTACGACAGCA	GGGTCTCCTTGTCAATTGTCC

NR0B1	TTTCTTTCCAAATGCTGGAGTCTGA	GAATGTACTTCACGCACTGCAG
NT5E	TGATGAACGCAACAATGGAATC	GGGTCATAACTGGGCACTCG
NTRK1	AGTCAGCCACGGTGATGAA	CACGTTCTTCTGTTGAGGTC
PGF	CAGACAAGGCCCACTGCT	GGCTGTTCCCTTGCTTCC
PHLDA1	GGGCAAGACAAGGTTTTGAGG	GGGGCGGAGAGACTGTTTTG
PHOSPHO1	CCCCACTTCTTACACTCCAAA	AGCCGTCGTCACACGTTCC
PPP1R1A	CCCAGACACAGAAGTGGAGTC	GGGATGCATTCTGCAGTTTT
PRMT8	GTGGTGACCAATGCCTGTTT	GCTCTTCCGTCTTCACTGTGT
PRRT4	CAGCCAAGTCCTCTGGA	TGGGGACTGTGGGGTCTA
PRRX2	AGCCTCGTCCCCCTACAG	GTGCAGGCTGAACTCCTTG
PRSS23	TCGGCGCGGAACAGTG	TATGCAGGCCAAGTGGGTTT
PTX3	GCCGGCAGGTTGTGAAACAG	TGACCCAAATGCAGGCACTA
RAMP1	TCACCCAGTTCCAGGTAGACA	CAGCTCCCTGTAGCTCCTGA
RAP1GAP	GGAGGAGACGGAGGGTGT	GTGTTCCCTGAGGATGACACG
RARRES2	TGGAATATTTGTGAGGCTGGA	CAGGCATTTCCGTTTTCTC
RASD2	GCAGGCTGTCCATCCTCA	GCTTGACCTCATCGAAGGAC
RIPK3	GCCTCCACAGCCAGTGAC	TCGGTTGGCAACTCAACTT
SERPINE1	ATCCCCATCCTACGTGGC	ATCTGCTGCTGGGTTTTCTCC
SP6	CTCTTTTATCACCGCCACTTG	GCAGACAGCGGTTAGCATT
STAU1	TGTGAATTTGAGGTGGCCC	ACTCCCCAACCGAAACCTTG
TSPAN11	ATCCTCTGGGAGCGGAAG	GGAAGATGACGAGCAACAGG
UPP1	AGTCACAATGATTGCCCGT	GGCTGGGAAATTGTGTCTGC
WFDC1	GCCCTGAGGAGGTGTTACAA	GGGACAGAGCAGGGGTTCC
ZNF467	GCTCAGCACAGAAGGCTCA	CTTCACCTTCCGAATCATCC

Northern Probe Primers

#1 1for/1rev	TGGCTCCCTTTGAAGAACTGCC	ATTGCCAGGGGTCTCGGTG
#2 2for/2rev	GAGCCAGCAGAGCTCAGGCCACAGC	CCCAAGAAGAGGGGCTCTGAAA
#3 3for/3rev	TTTCAGAGAGCCCCTCTTCTTGGG	CTTCTTGCCATTTGGAGGTG
#4 4for/4rev	CACCTCCAAATGGCCAAGAAG	CATTGTGCTCCGGGCTCAGC

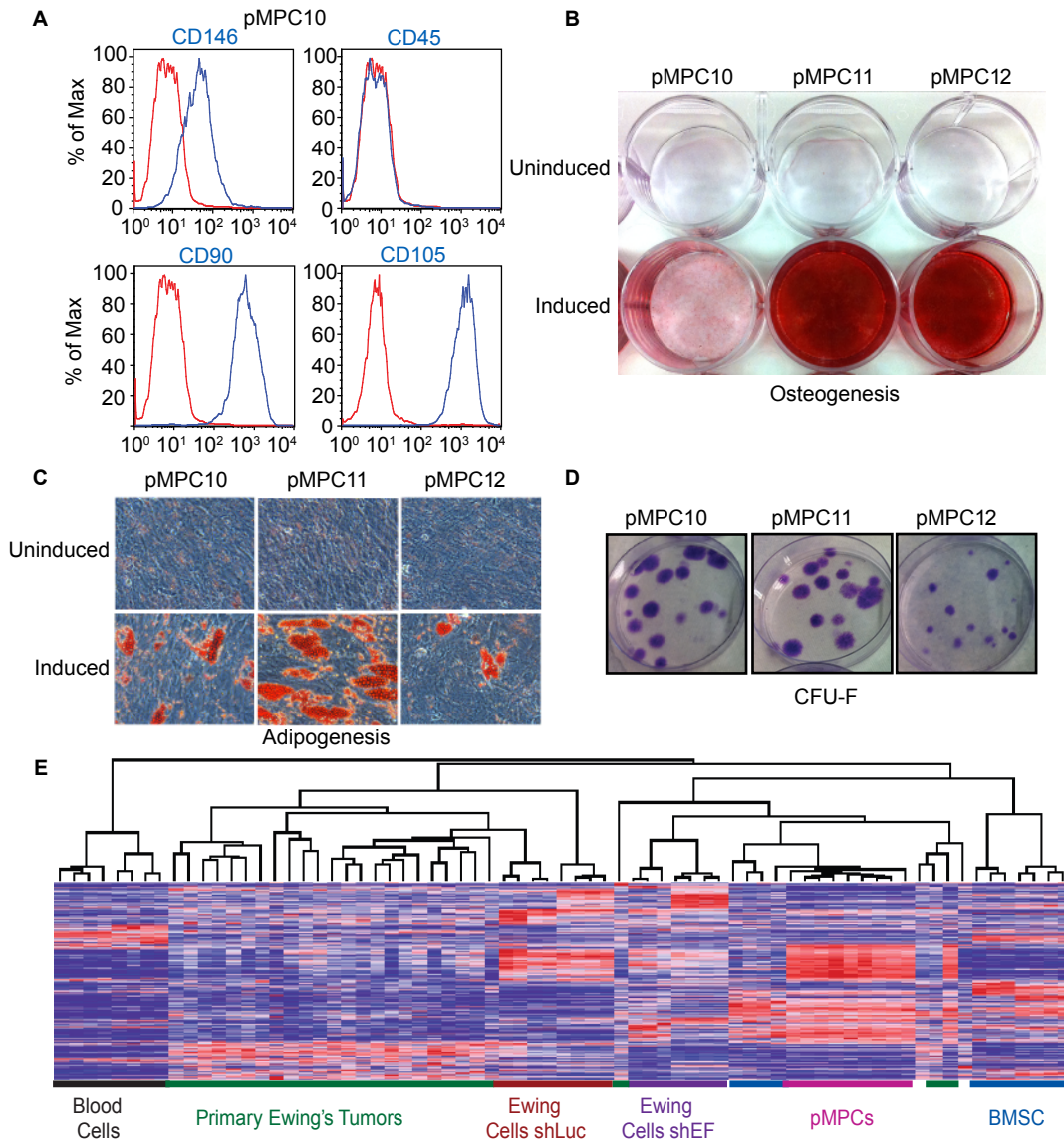
Cloning Primers

oe clone for	ACAACTATGGCTTGTCTGAGTCCTG
oe clone rev	ATGTTTTGAGCTCTTTATTTGATGTGC

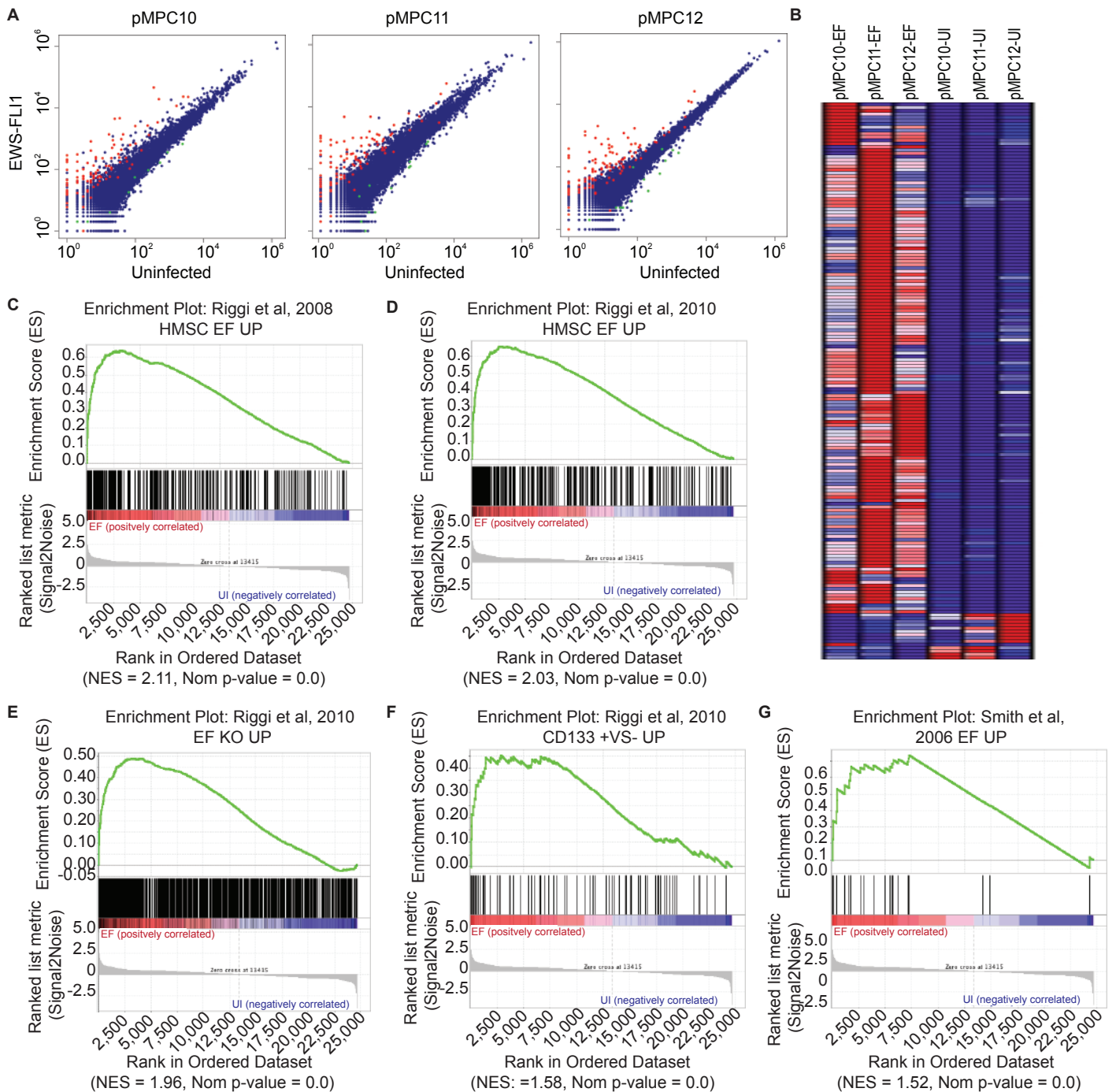
Isoform Primers

1for	TCCAAGACGCGTTTCAGAG
1rev	CCACAAAGAGTTTACCAAGGTGT
2for	CCTGCGCTGGGGAGTTGGTG
2rev	GCAGGCCCAAGAAGAGGGGC
3rev	AGGAAATGGATGGACTCCACCTCC

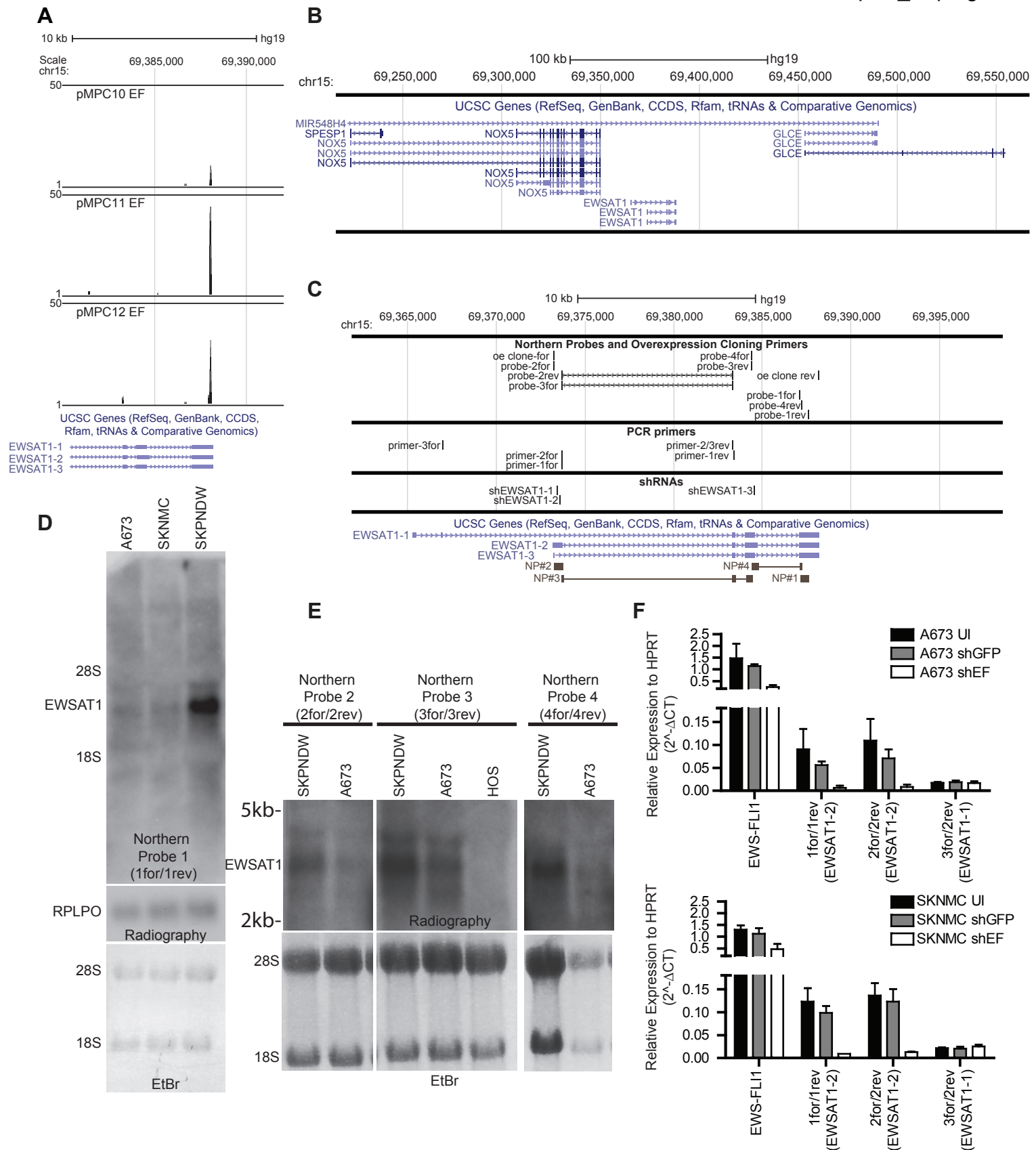




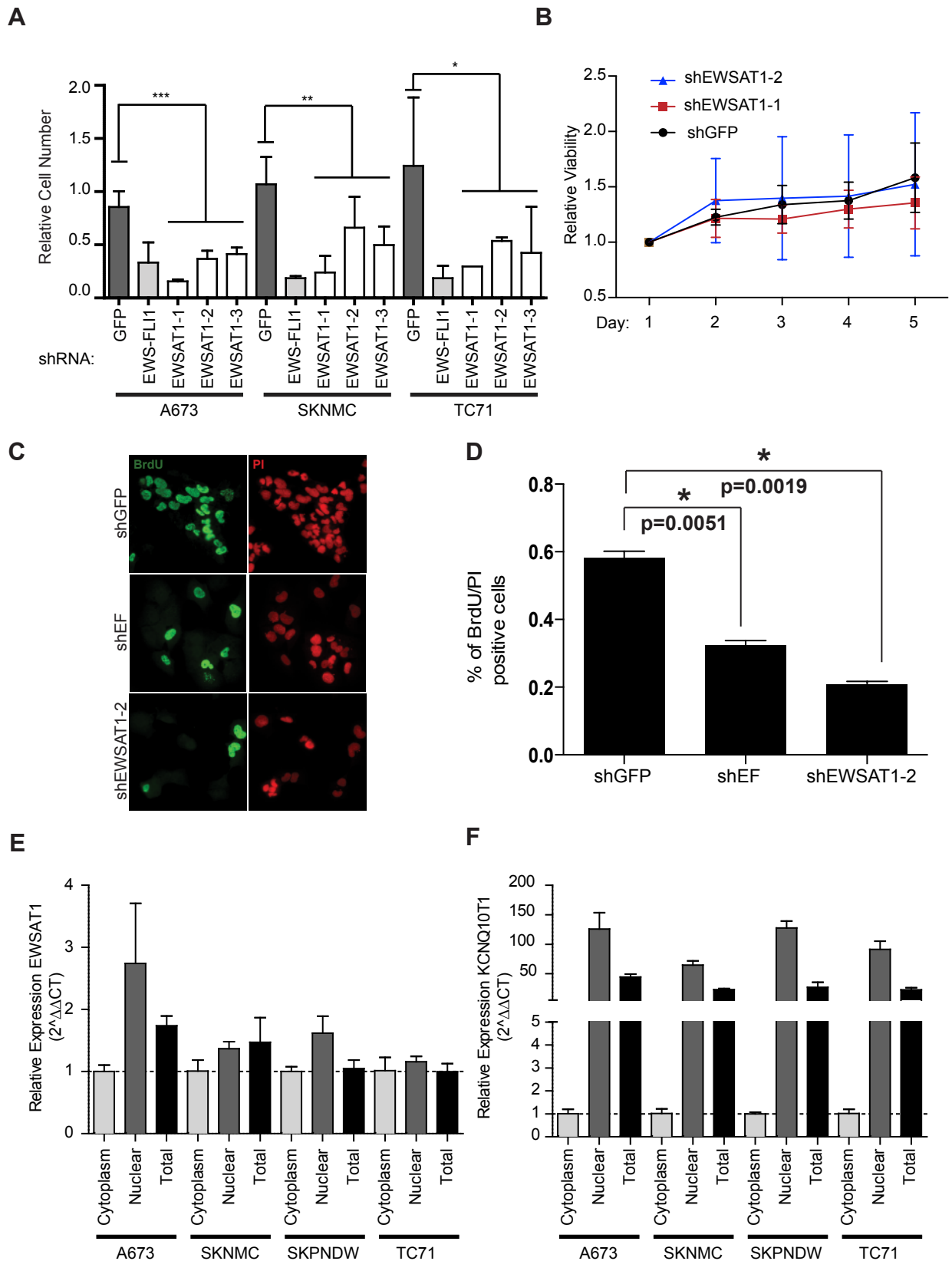
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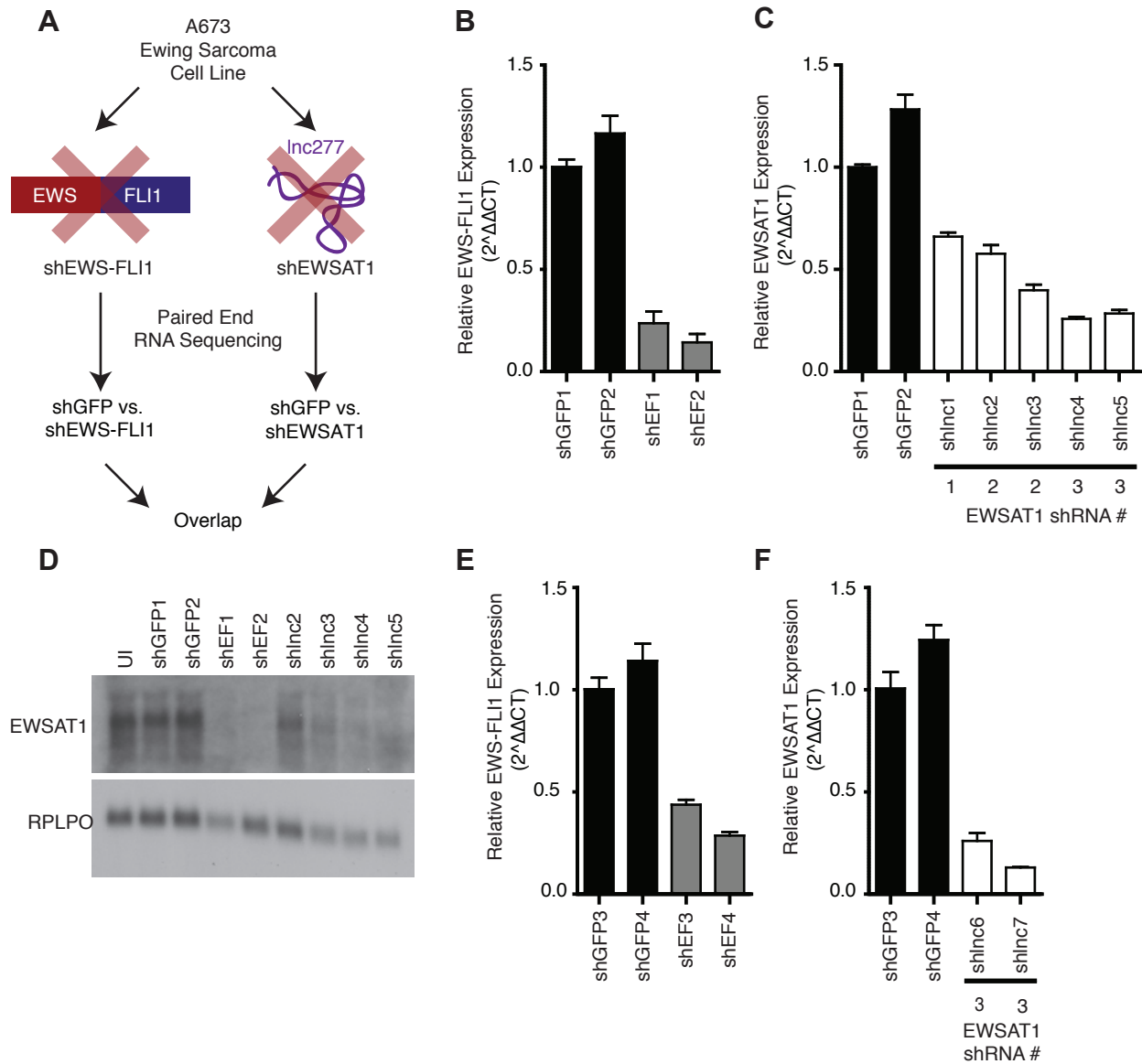
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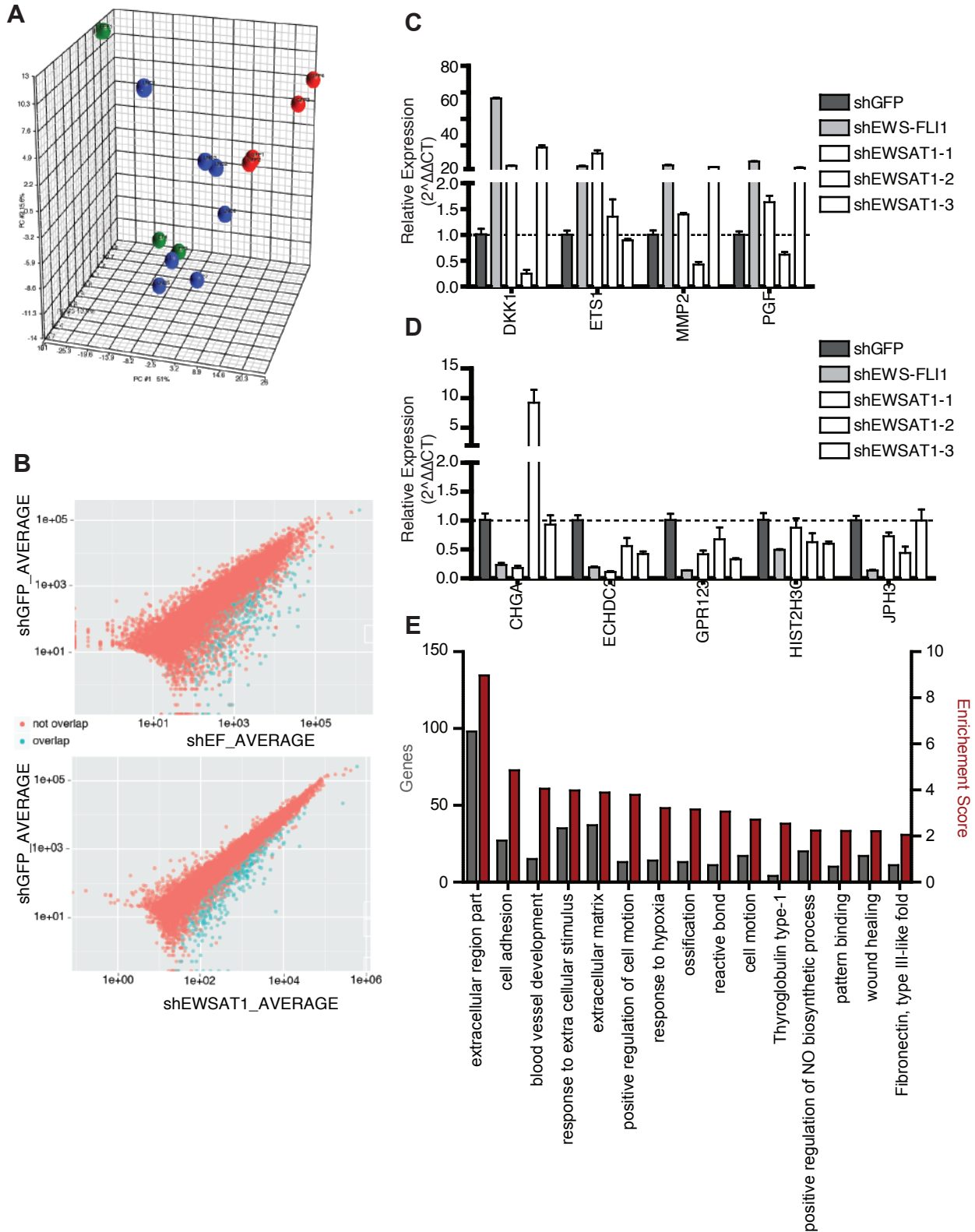
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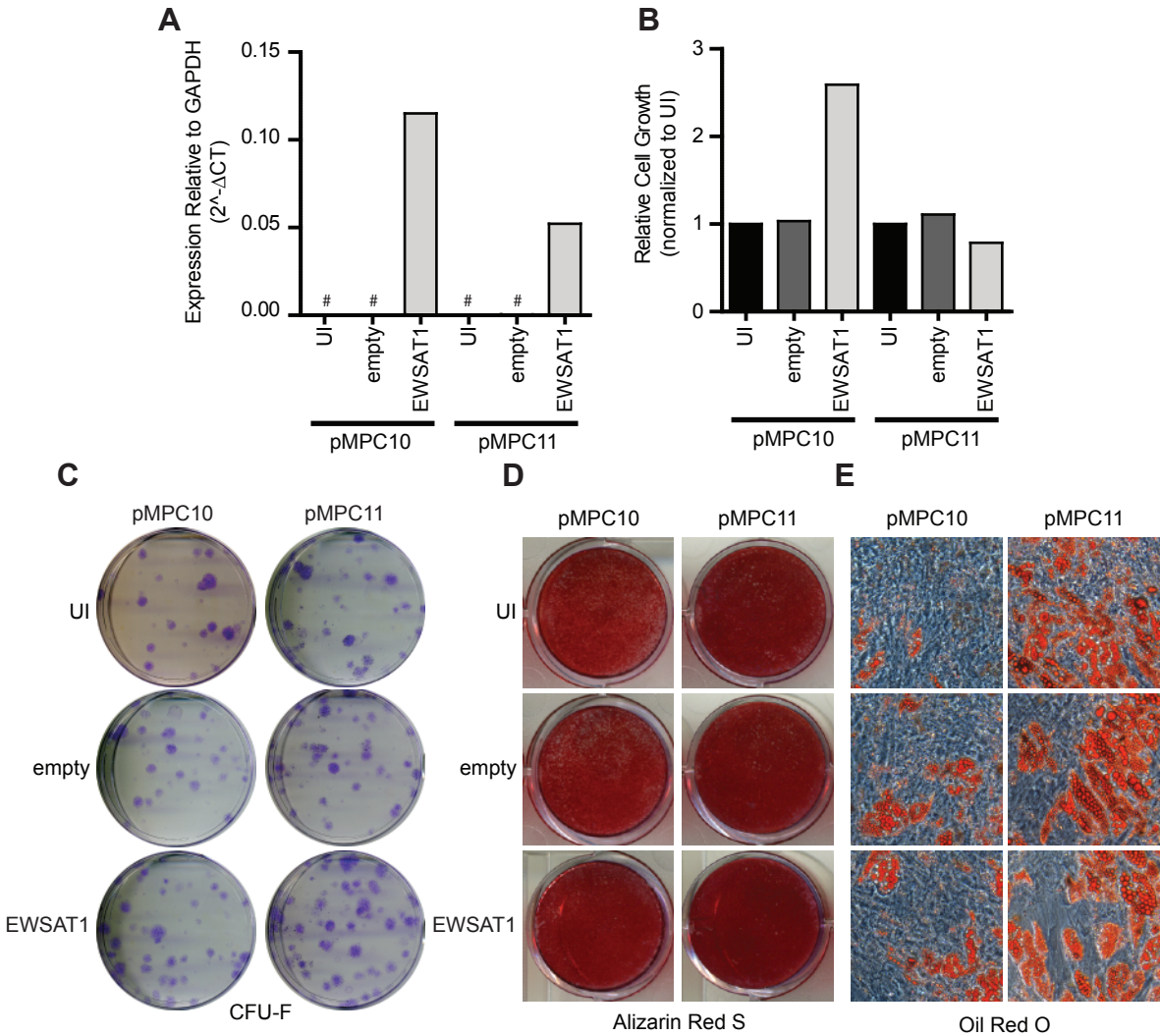
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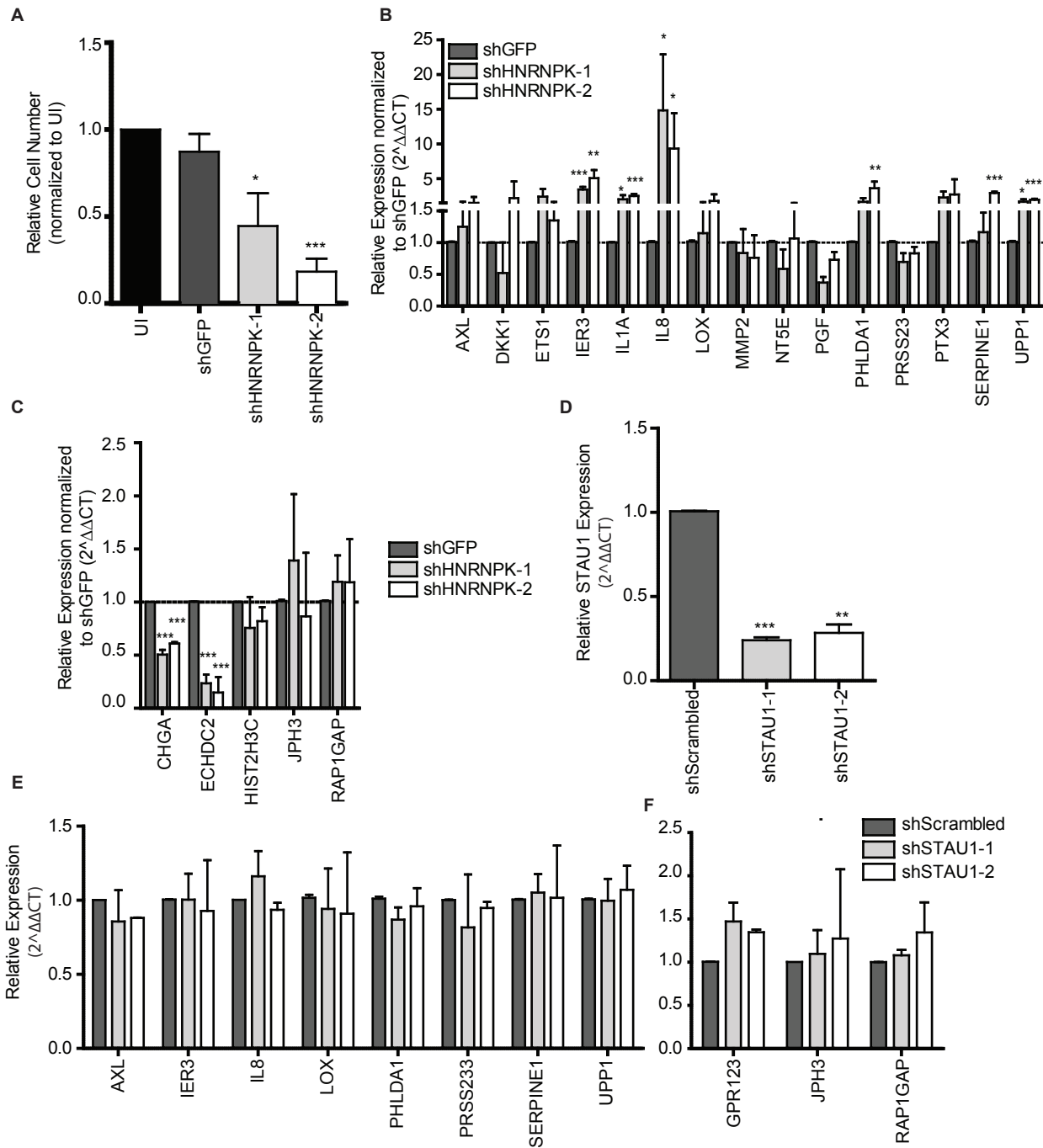
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**Supplemental Figure 7.** EWSAT1 overexpression does not consistently alter the growth and differentiation of pMPC cells. A. RT-PCR showing expression of EWSAT1 in pMPC cells. EWSAT1 expression is relative to GAPDH. # = not expressed. B. Cell growth of pMPCs expressing EWSAT1. C. CFU-F formation of pMPCs expressing EWSAT1. D. Osteogenic differentiation of pMPCs expressing EWSAT1. E. Adipogenic differentiation of pMPCs expressing EWSAT1.



**Supplemental Figure 8.** HNRNPK is required for cell growth and STAU1 knockdown does not effect the gene expression of EWS-FLI1 and EWSAT1 overlap genes. A. Cell growth assay in A673 cells transduced with shRNAs normalized to uninfected cells (UI). Error bars represent standard deviation for n=3. B. RT-PCR on genes that are repressed by EWS-FLI1 and EWSAT1 in A673 cells with knockdown of hnrNPK normalized to shGFP. C. RT-PCR on genes that are induced by EWS-FLI1 and EWSAT1 in A673 cells with knockdown of HNRNPK normalized to shGFP. \*\*\* p-value < 0.001 \*\* p-value < 0.01, \* p-value < 0.05 by two-tailed t-tests. D. RT-PCR on STAU1. Normalized to shScrambled. E. RT-PCR on genes found to be repressed by EWS-FLI1 and EWSAT1. Normalized to shScrambled. F. RT-PCR on genes found to be induced by EWS-FLI1 and EWSAT1. Normalized to shScrambled. Error bars represent standard deviation for n=2. \*\*\* p-value < 0.001 and \*\* p-value < 0.01 by two-tailed t-test.