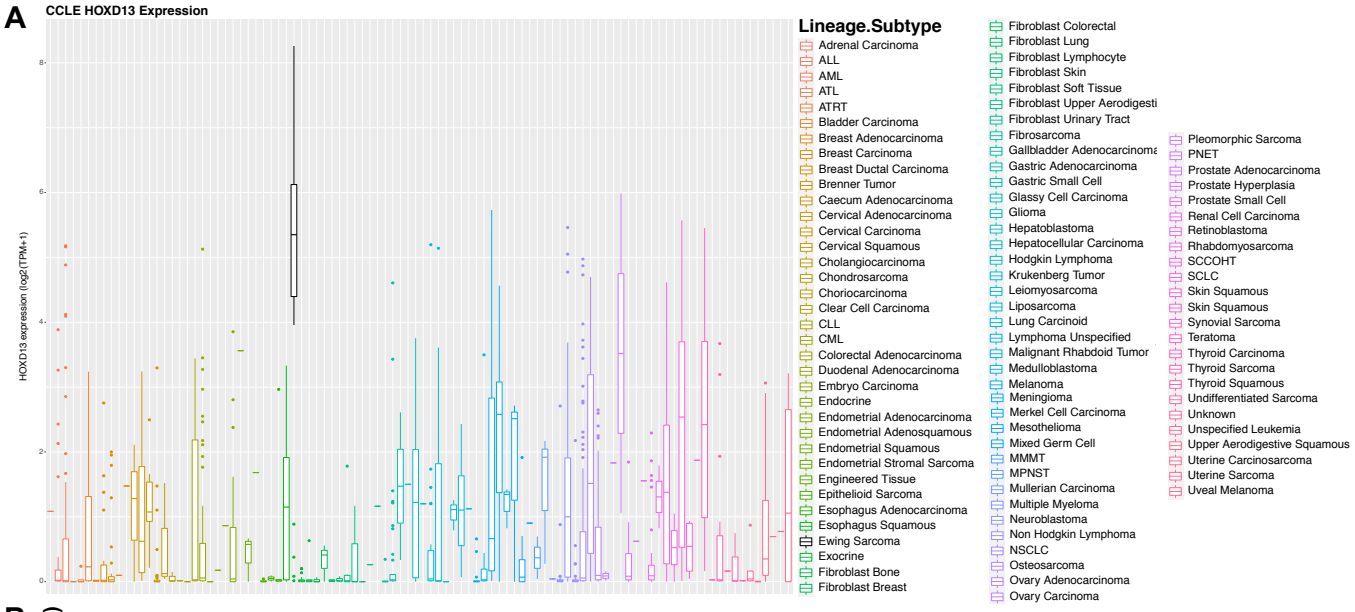
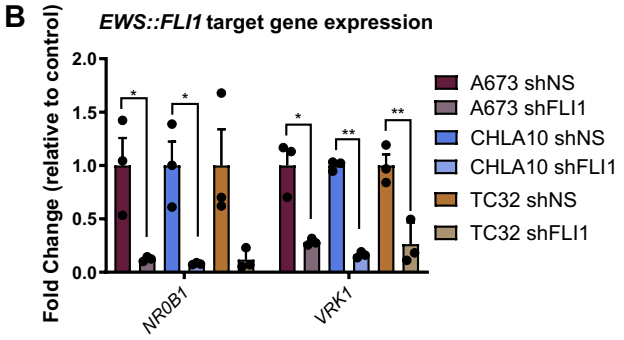
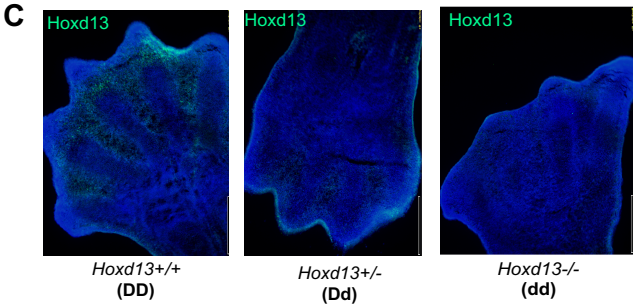
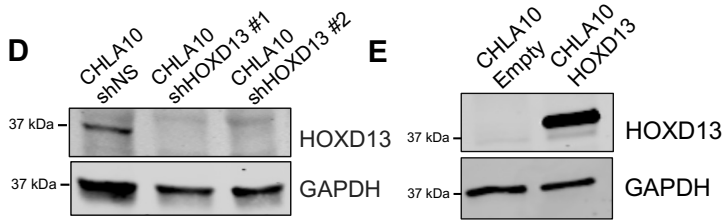
Supplementary Figures

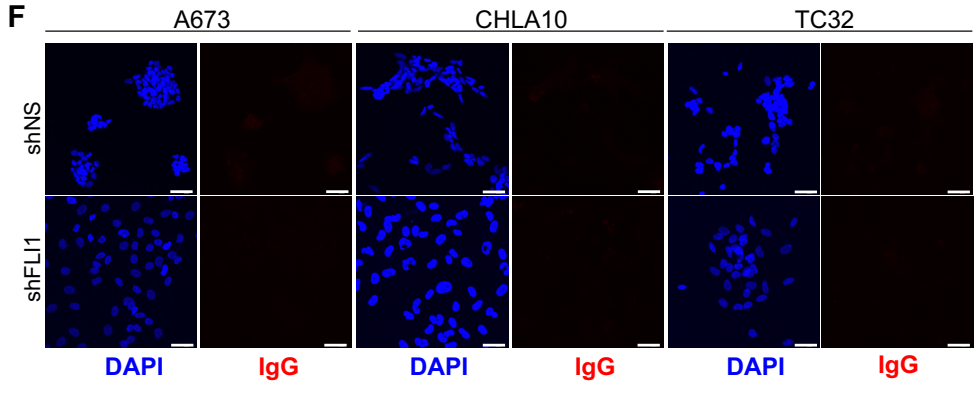
Figure S1

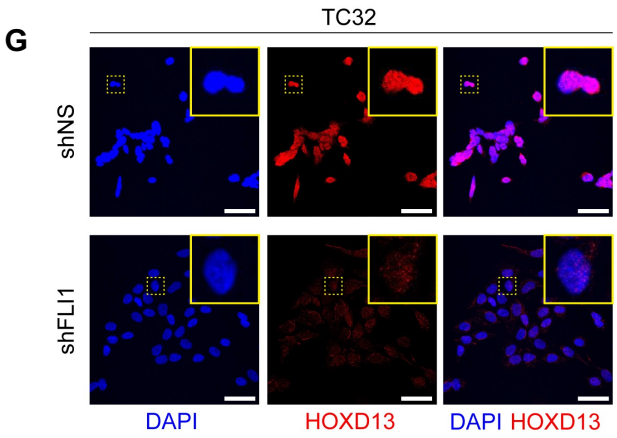












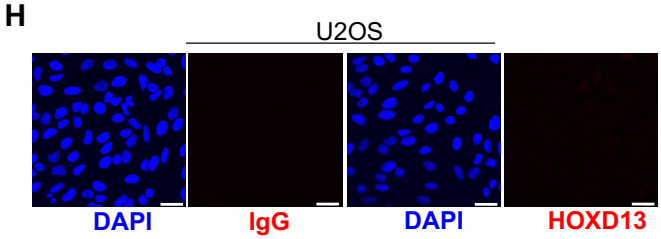


Figure S1. Authentication of EWS::FLI1 knockdown cells and the HOXD13 antibody. A) *HOXD13* expression across different cell lines grouped by lineage subtype, EwS is in black (32). B) qRT-PCR of EWS::FLI1 target genes, *NR0B1* and *VRK1* after knockdown. Error bars representative of SEM from three independent replicates. * $p<0.05$; ** $p<0.01$; Two-way ANOVA; Sidak's multiple comparison test. C) Immunofluorescence detection of Hoxd13 protein expression in distal limbs (autopods) derived from E13.5 *Hoxd13* WT (+/+), het (+/-), or knockout (-/-) mouse embryos. Western blot for HOXD13 after D) knockdown (100ug protein) and E) overexpression (30 ug protein) in CHLA10 cells. F) Fluorescent Immunocytochemistry of HOXD13 (Alexa647) in EWS::FLI1 knockdown cells. Nuclear counterstain was performed with DAPI. Scale bar is 50 um. G-H) Immunofluorescent staining of shFLI1 and U2OS cells with IgG isotype control and HOXD13 antibodies, respectively (secondary antibody Alexa647). Nuclear counterstain was performed with DAPI. Scale bar is 50 um.

Figure S2

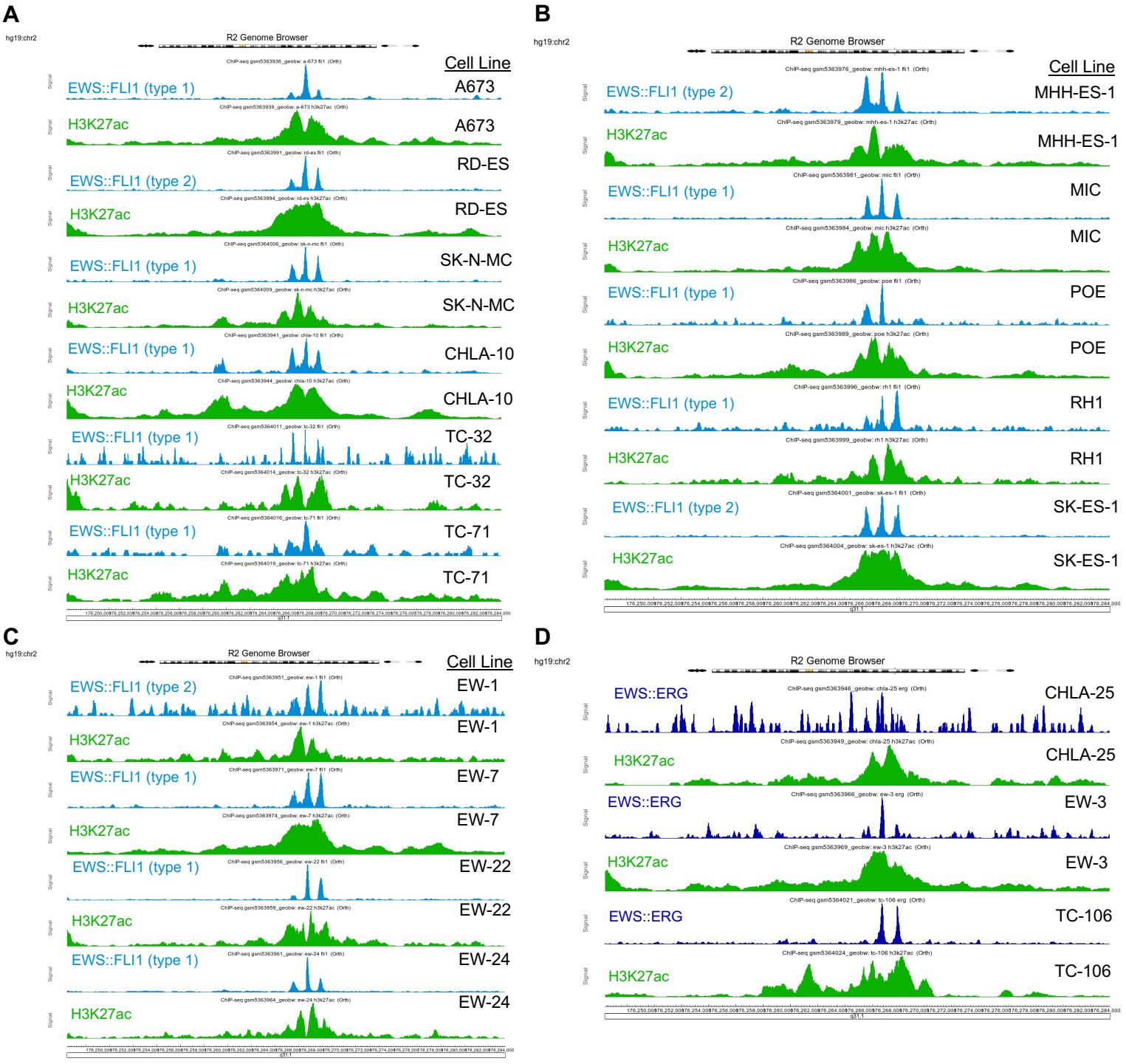

A
hg19:chr2
R2 Genome Browser
Cell Line
EWS::FLI1 (type 1)
A673
H3K27ac
A673
EWS::FLI1 (type 2)
RD-ES
H3K27ac
RD-ES
EWS::FLI1 (type 1)
SK-N-MC
H3K27ac
SK-N-MC
EWS::FLI1 (type 1)
CHLA-10
H3K27ac
CHLA-10
EWS::FLI1 (type 1)
TC-32
H3K27ac
TC-32
EWS::FLI1 (type 1)
TC-71
H3K27ac
TC-71
B
hg19:chr2
R2 Genome Browser
Cell Line
EWS::FLI1 (type 2)
MHH-ES-1
H3K27ac
MHH-ES-1
EWS::FLI1 (type 1)
MIC
H3K27ac
MIC
EWS::FLI1 (type 1)
POE
H3K27ac
POE
EWS::FLI1 (type 1)
RH1
H3K27ac
RH1
EWS::FLI1 (type 2)
SK-ES-1
H3K27ac
SK-ES-1
C
hg19:chr2
R2 Genome Browser
Cell Line
EWS::FLI1 (type 2)
EW-1
H3K27ac
EW-1
EWS::FLI1 (type 1)
EW-7
H3K27ac
EW-7
EWS::FLI1 (type 1)
EW-22
H3K27ac
EW-22
EWS::FLI1 (type 1)
EW-24
H3K27ac
EW-24
D
hg19:chr2
R2 Genome Browser
EWS::ERG
CHLA-25
H3K27ac
CHLA-25
EWS::ERG
EW-3
H3K27ac
EW-3
EWS::ERG
TC-106
H3K27ac
TC-106

Figure S2. EWS::ETS binding and H3K27ac is conserved across EwS cell lines. A-C) ChIP-seq tracks of EWS::FLI1 binding (blue) and H3K27ac (green) at the PHE of EwS cells (34,37). D) ChIP-seq tracks of EWS::ERG binding and H3K27ac at the PHE of EwS cells (34,37).

Figure S3

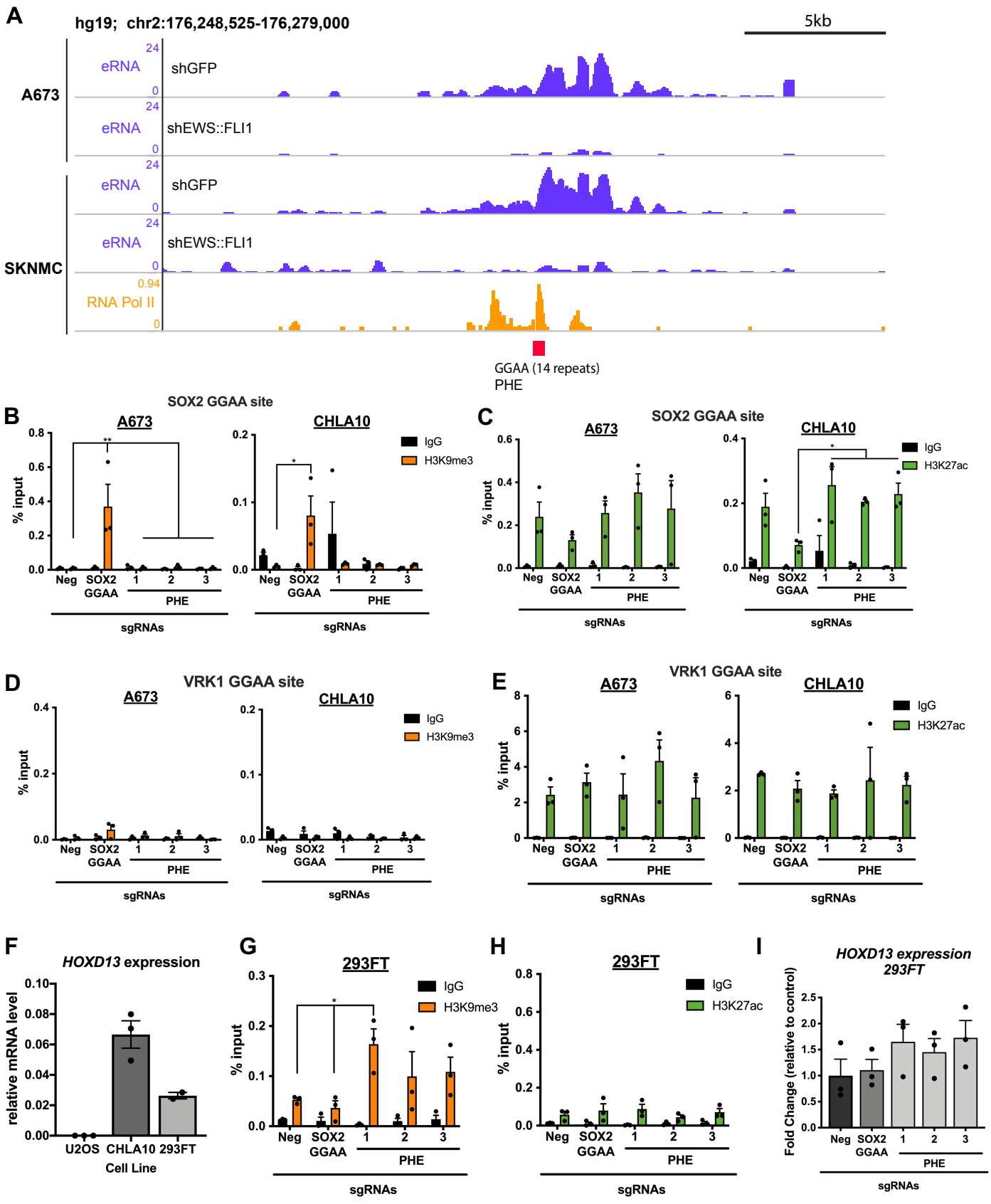

A
hg19; chr2:176,248,525-176,279,000
5kb
A673
SKNMC
eRNA
shGFP
shEWS::FLI1
RNA Pol II
GGAA (14 repeats)
PHE
B
SOX2 GGAA site
A673
CHLA10
IgG
H3K9me3
% input
Neg
SOX2 GGAA
PHE
sgRNAs
C
SOX2 GGAA site
H3K27ac
D
VRK1 GGAA site
E
VRK1 GGAA site
F
HOXD13 expression
relative mRNA level
U2OS
CHLA10
293FT
Cell Line
G
293FT
H
293FT
I
HOXD13 expression
293FT
Fold Change (relative to control)

Figure S3. Enhancer RNA expression at the PHE. A) NRO-seq tracks of eRNA transcription at the PHE region in A673 and SKNMC cells (6). RNA polymerase II (RNA Pol II) binding at the PHE region in SKNMC cells (ENCODE project GSE32465). ChIP-qPCR for **B)** H3K9me3 and **C)** H3K27ac at the SOX2 GGAA enhancer site. ChIP-qPCR for **D)** H3K9me3 and **E**) H3K27ac at the VRK1 GGAA enhancer site. VRK1 enhancer used as a control GGAA site. **F)** qRT-PCR of baseline *HOXD13* expression in 293FT, U2OS, and CHLA10 cells. ChIP-qPCR for **G)** H3K9me3 and **H)** H3K27ac at the PHE region in gRNA-targeted 293FT cells. **I)** qRT-qPCR of *HOXD13* expression in PHE-targeted 293FT cells. Error bars are representative of SEM from at least three independent experiments. * $p<0.05$; ** $p<0.01$; Two tailed t-test; Two-way ANOVA; Sidak's multiple comparison test.

Figure S4

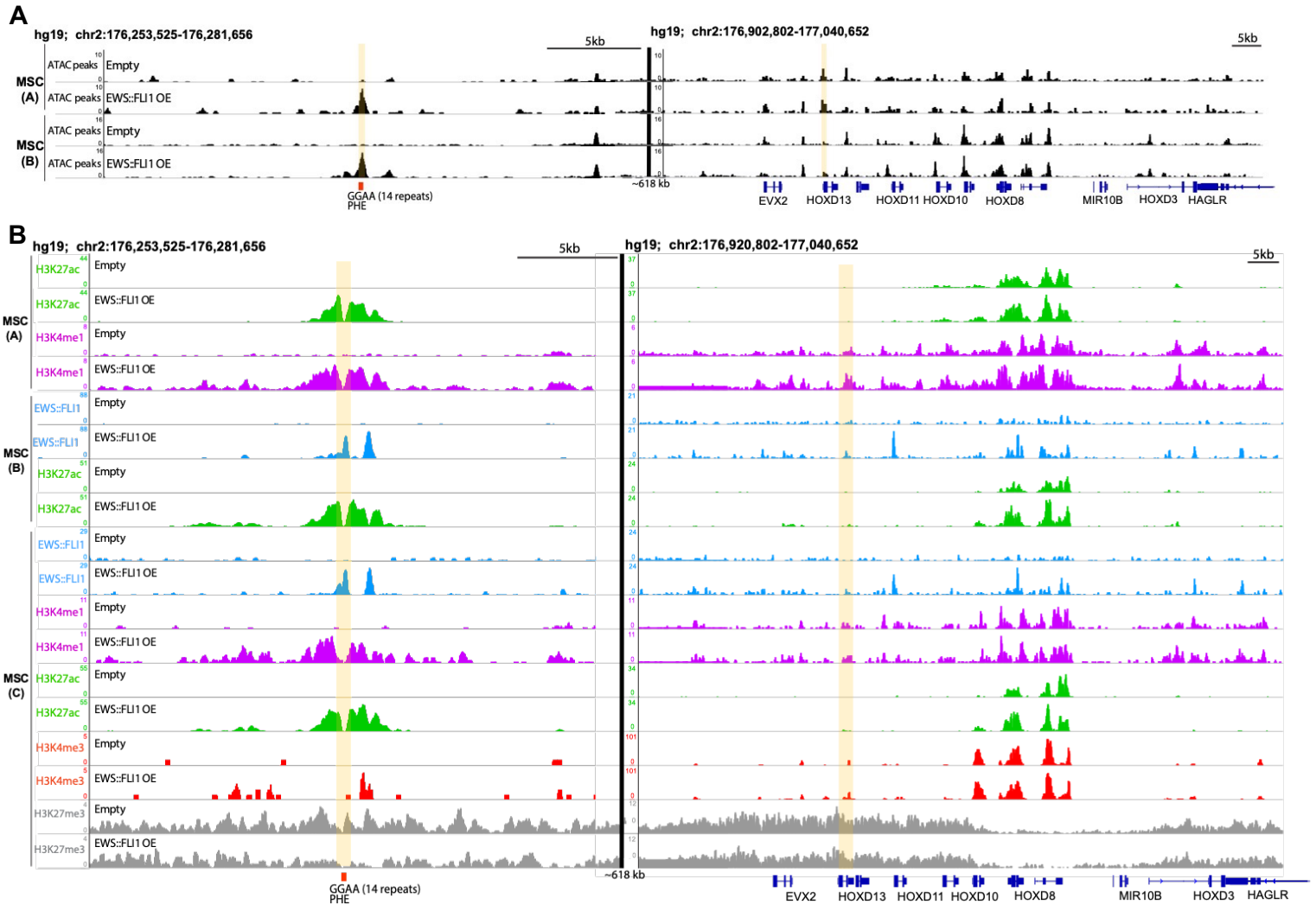

A
hg19; chr2:176,253,525-176,281,656
5kb
hg19; chr2:176,902,802-177,040,652
5kb
MSC (A)
MSC (B)
ATAC peaks
Empty
EWS::FLI1 OE
~618 kb
GGAA (14 repeats)
PHE
EVX2
HOXD13
HOXD11
HOXD10
HOXD8
MIR10B
HOXD3
HAGLR
B
hg19; chr2:176,253,525-176,281,656
hg19; chr2:176,920,802-177,040,652
MSC (A)
MSC (B)
MSC (C)
H3K27ac
H3K4me1
H3K4me3
H3K27me3
EWS::FLI1
Empty
EWS::FLI1 OE
C
H7 MSC
D
U2OS
E
SW1353
EWS::FLI1
HOXD13
relative mRNA level
Empty
EWS::FLI1
A673

Figure S4. EWS::FLI1 is not sufficient to induce *HOXD13* transcription in heterologous cell types. A) ATAC-seq tracks at the PHE and HOXD locus in MSCs transduced with EWS::FLI1 or an empty vector. B) ChIP-seq tracks for EWS::FLI1, H3K27ac, H3K4me1, H3K4me3, and H3K27me3 at the PHE and HOXD locus in MSC's overexpressing EWS::FLI1 or an empty vector. MSC ATAC and ChIP-seq tracks from Refs. (A-C: 2,5,& 6, respectively). qRT-PCR measurement of *EWS::FLI1* and *HOXD13* expression in C) H7 MSCs, D) U20S, and E) SW1353 cells after EWS::FLI1 transduction. A673 cells shown for comparison.

Figure S5

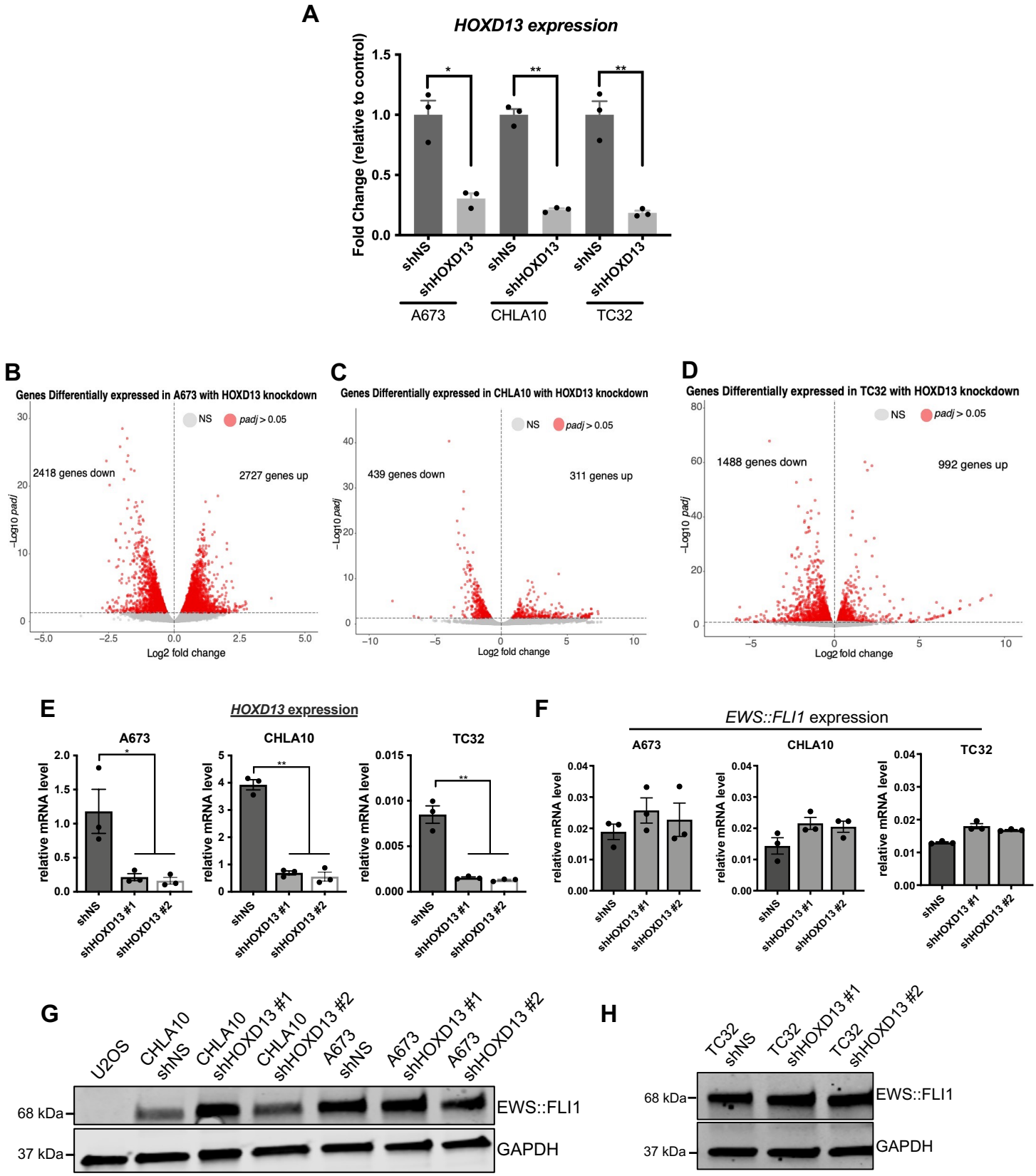

A
HOXD13 expression
Fold Change (relative to control)
shNS
shHOXD13
A673
CHLA10
TC32
B
Genes Differentially expressed in A673 with HOXD13 knockdown
2418 genes down
2727 genes up
Log2 fold change
C
Genes Differentially expressed in CHLA10 with HOXD13 knockdown
439 genes down
311 genes up
D
Genes Differentially expressed in TC32 with HOXD13 knockdown
1488 genes down
992 genes up
E
HOXD13 expression
relative mRNA level
shHOXD13 #1
shHOXD13 #2
F
EWS::FLI1 expression
G
U2OS
CHLA10 shNS
CHLA10 shHOXD13 #1
CHLA10 shHOXD13 #2
A673 shNS
A673 shHOXD13 #1
A673 shHOXD13 #2
68 kDa
EWS::FLI1
37 kDa
GAPDH
H
TC32 shNS
TC32 shHOXD13 #1
TC32 shHOXD13 #2

Figure S5. HOXD13-regulated gene programs are largely cell line-dependent. A) qRT-PCR of *HOXD13* expression in cells analyzed by RNAseq. B-D) Volcano plots for each cell line depicting the differentially expressed genes after HOXD13 knockdown (*padj <0.05*). E) qRT-PCR of *HOXD13* expression of control (shNS) and *HOXD13* (shHOXD13) knockdown cells. F) qRT-PCR of EWS::FLI1 expression in HOXD13 knockdown cells. G-H) Western blot of EWS::FLI1 expression in HOXD13 knockdown cells. Two-tailed *t*-test; One-way ANOVA followed by a Tukey's multiple comparison test. Error bars representative of SEM from three independent replicates. * $p<0.05$*;* ** $p<0.01$; Two-way ANOVA; Sidak's multiple comparison test.

Figure S6

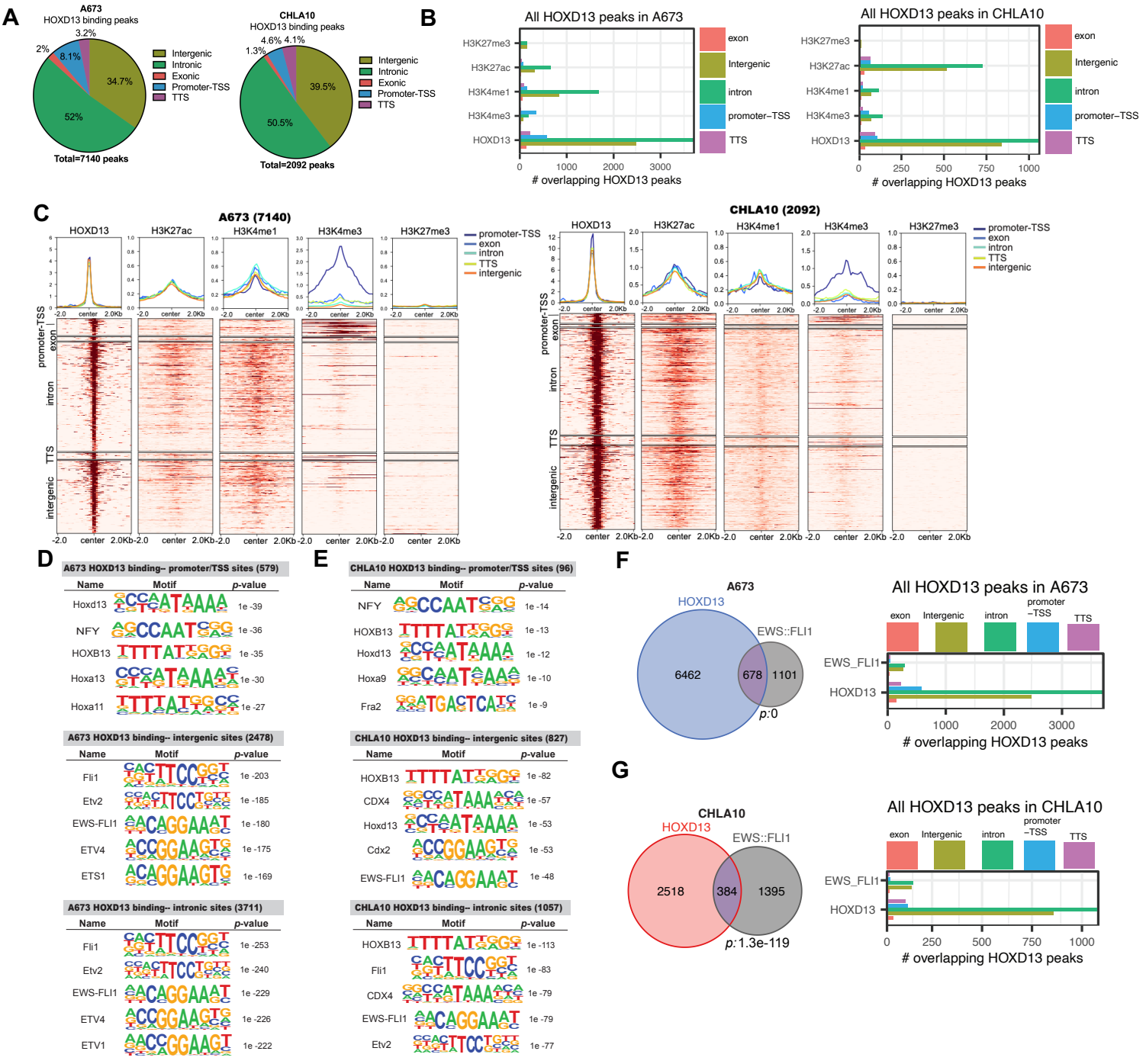

A
A673
HOXD13 binding peaks
3.2%
2%
8.1%
34.7%
52%
Intergenic
Intronic
Exonic
Promoter-TSS
TTS
Total=7140 peaks
CHLA10
HOXD13 binding peaks
4.6% 4.1%
1.3%
39.5%
50.5%
Intergenic
Intronic
Exonic
Promoter-TSS
TTS
Total=2092 peaks
B
All HOXD13 peaks in A673
H3K27me3
H3K27ac
H3K4me1
H3K4me3
HOXD13
exon
Intergenic
intron
promoter−TSS
TTS
0
1000
2000
3000
overlapping HOXD13 peaks
All HOXD13 peaks in CHLA10
H3K27me3
H3K27ac
H3K4me1
H3K4me3
HOXD13
exon
Intergenic
intron
promoter−TSS
TTS
0
250
500
750
1000
overlapping HOXD13 peaks
C
A673 (7140)
HOXD13
H3K27ac
H3K4me1
H3K4me3
H3K27me3
promoter-TSS
exon
intron
TTS
intergenic
-2.0
center
2.0Kb
CHLA10 (2092)
HOXD13
H3K27ac
H3K4me1
H3K4me3
H3K27me3
promoter-TSS
exon
intron
TTS
intergenic
-2.0
center
2.0Kb
D
A673 HOXD13 binding-- promoter/TSS sites (579)
Name
Motif
p-value
Hoxd13
1e -39
NFY
1e -36
HOXB13
1e -35
Hoxa13
1e -30
Hoxa11
1e -27
A673 HOXD13 binding-- intergenic sites (2478)
Name
Motif
p-value
Fli1
1e -203
Etv2
1e -185
EWS-FLI1
1e -180
ETV4
1e -175
ETS1
1e -169
A673 HOXD13 binding-- intronic sites (3711)
Name
Motif
p-value
Fli1
1e -253
Etv2
1e -240
EWS-FLI1
1e -229
ETV4
1e -226
ETV1
1e -222
E
CHLA10 HOXD13 binding-- promoter/TSS sites (96)
Name
Motif
p-value
NFY
1e -14
HOXB13
1e -13
Hoxd13
1e -12
Hoxa9
1e -10
Fra2
1e -9
CHLA10 HOXD13 binding-- intergenic sites (827)
Name
Motif
p-value
HOXB13
1e -82
CDX4
1e -57
Hoxd13
1e -53
Cdx2
1e -53
EWS-FLI1
1e -48
CHLA10 HOXD13 binding-- intronic sites (1057)
Name
Motif
p-value
HOXB13
1e -113
Fli1
1e -83
CDX4
1e -79
EWS-FLI1
1e -79
Etv2
1e -77
F
A673
HOXD13
EWS::FLI1
6462
678
1101
p:0
All HOXD13 peaks in A673
exon
Intergenic
intron
promoter −TSS
TTS
EWS_FLI1
HOXD13
0
1000
2000
3000
overlapping HOXD13 peaks
G
CHLA10
HOXD13
EWS::FLI1
2518
384
1395
p:1.3e-119
All HOXD13 peaks in CHLA10
exon
Intergenic
intron
promoter −TSS
TTS
EWS_FLI1
HOXD13
0
250
500
750
1000
overlapping HOXD13 peaks

Figure S6. HOXD13 binding distribution is similar in A673 and CHLA10 cells. A) Pie chart showing the genomic distribution HOXD13 binding sites in A673 and CHLA10 cells. B) Bar chart summarizing shared binding sites and associated histone marks at these sites. C) Tornado plots depicting shared HOXD13 binding by genomic location and the associated histone marks at these sites. HOMER Motif analysis by genomic location for the HOXD13 binding sites in D) A673 and E) CHLA10 cells. Venn diagrams showing the overlap between HOXD13 binding sites and published EWS::FLI1 binding sites (2) in F) A673 and G) CHLA10.

Figure S7

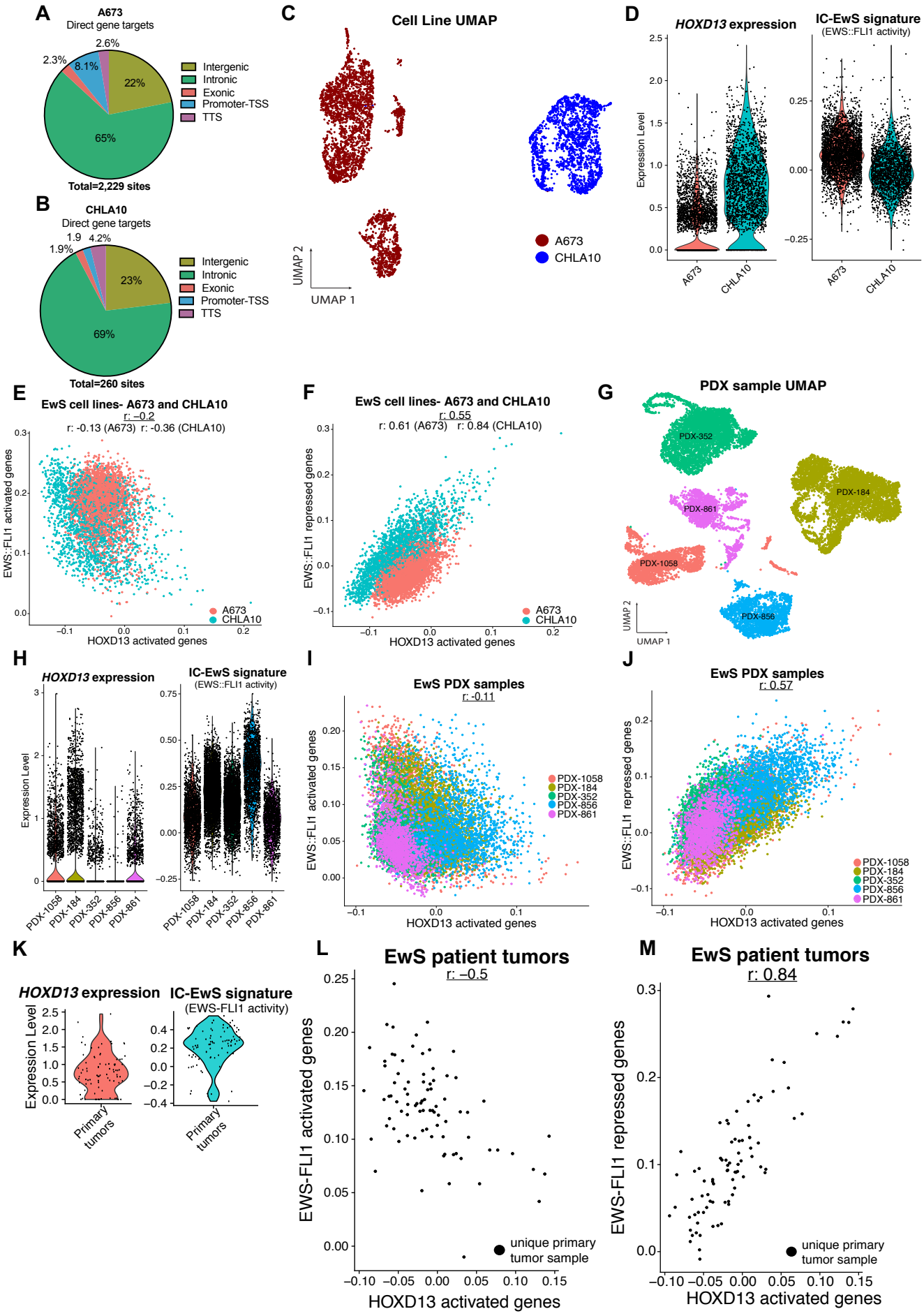

A
A673
Direct gene targets
2.6%
2.3%
8.1%
22%
65%
Intergenic
Intronic
Exonic
Promoter-TSS
TTS
Total=2,229 sites
B
CHLA10
Direct gene targets
1.9
4.2%
1.9%
23%
69%
Intergenic
Intronic
Exonic
Promoter-TSS
TTS
Total=260 sites
C
Cell Line UMAP
UMAP 2
UMAP 1
A673
CHLA10
D
HOXD13 expression
IC-EwS signature
(EWS::FLI1 activity)
Expression Level
A673
CHLA10
E
EwS cell lines- A673 and CHLA10
r: −0.2
r: -0.13 (A673) r: -0.36 (CHLA10)
EWS::FLI1 activated genes
HOXD13 activated genes
A673
CHLA10
F
EwS cell lines- A673 and CHLA10
r: 0.55
r: 0.61 (A673) r: 0.84 (CHLA10)
EWS::FLI1 repressed genes
HOXD13 activated genes
A673
CHLA10
G
PDX sample UMAP
PDX-352
PDX-184
PDX-861
PDX-1058
PDX-856
UMAP 2
UMAP 1
H
HOXD13 expression
IC-EwS signature
(EWS::FLI1 activity)
Expression Level
PDX-1058
PDX-184
PDX-352
PDX-856
PDX-861
I
EwS PDX samples
r: -0.11
EWS::FLI1 activated genes
HOXD13 activated genes
PDX-1058
PDX-184
PDX-352
PDX-856
PDX-861
J
EwS PDX samples
r: 0.57
EWS::FLI1 repressed genes
HOXD13 activated genes
PDX-1058
PDX-184
PDX-352
PDX-856
PDX-861
K
HOXD13 expression
IC-EwS signature
(EWS-FLI1 activity)
Expression Level
Primary tumors
Primary tumors
L
EwS patient tumors
r: −0.5
EWS-FLI1 activated genes
HOXD13 activated genes
unique primary tumor sample
M
EwS patient tumors
r: 0.84
EWS-FLI1 repressed genes
HOXD13 activated genes
unique primary tumor sample

Figure S7. Single-cell expression of EWS::FLI1 and HOXD13 regulated gene sets. **A-B)** Pie charts depict the genomic distribution of these sites. Data were generated using CITE-seq. **C)** Low dimensional embedding, Uniform Manifold Approximation Projection (UMAP), of single-cell gene expression profiles of A673 and CHLA10 cells. **D)** Violin plots showing single-cell HOXD13 expression and the IC-EwS EWS::FLI1 signature in A673 and CHLA10 cells. Scatter plot of HOXD13 activated genes versus **E)** EWS::FLI1 activated genes and **F)** EWS::FLI1 repressed genes by cell line. **G)** Low dimensional embedding, Uniform Manifold Approximation Projection (UMAP), of single-cell gene expression profiles of 5 Ewing sarcoma PDX lines (35). **H)** Violin plots showing single-cell HOXD13 expression and the IC-EwS EWS::FLI1 signature in the PDX lines. Scatter plot of HOXD13 activated genes versus **I)** EWS::FLI1 activated genes and **J)** EWS::FLI1 repressed genes by PDX line. **K)** Violin plots showing HOXD13 expression and the IC-EwS EWS::FLI1 signature in primary tumor samples (n=84). Scatter plot of HOXD13 activated genes versus **L)** EWS::FLI1 activated genes and **M)** EWS::FLI1 repressed genes by primary tumor sample.