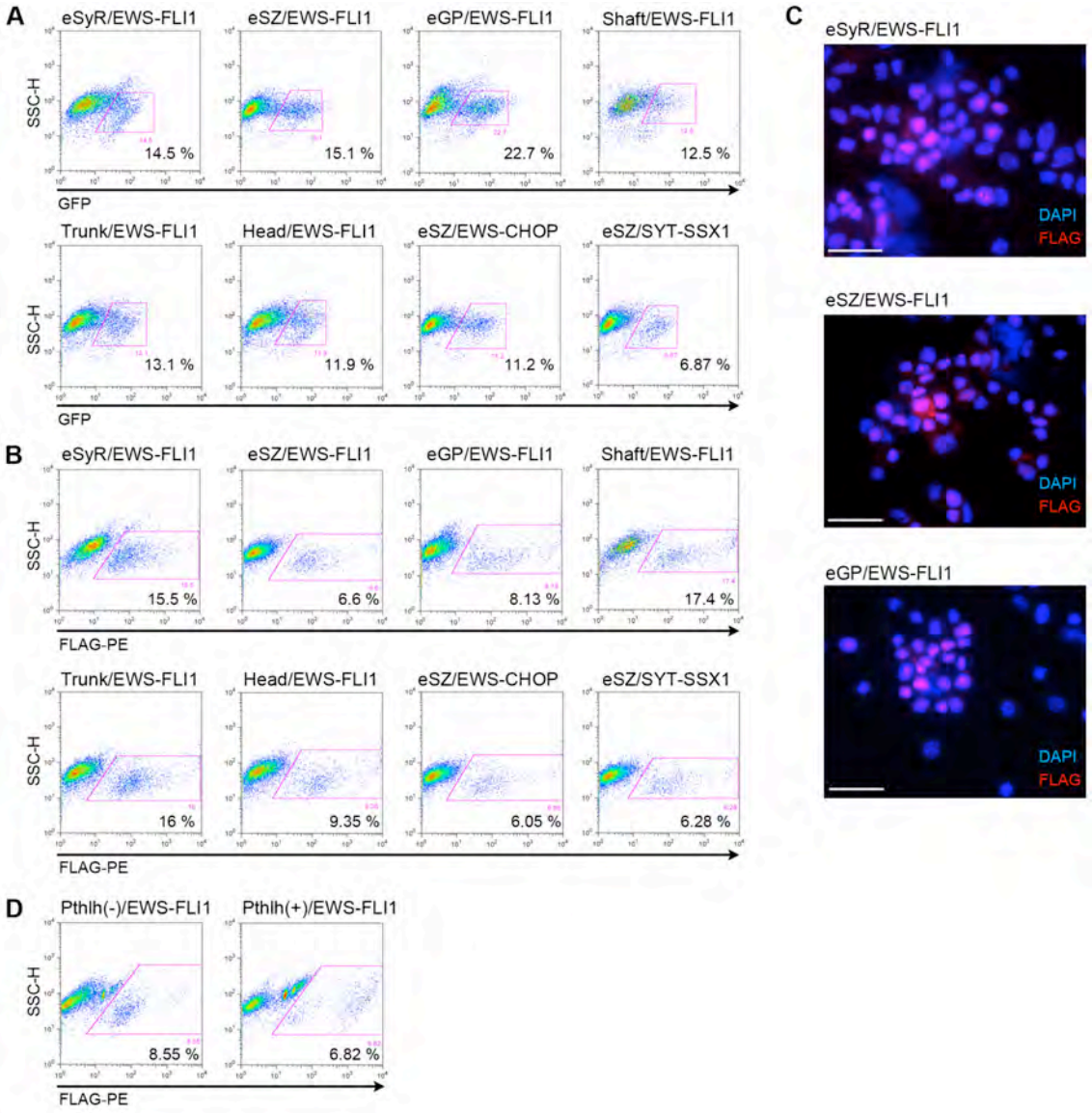
Supplemental Figures and Information

Ewing's sarcoma cell-of-origin is highly enriched in embryonic osteochondrogenic progenitors

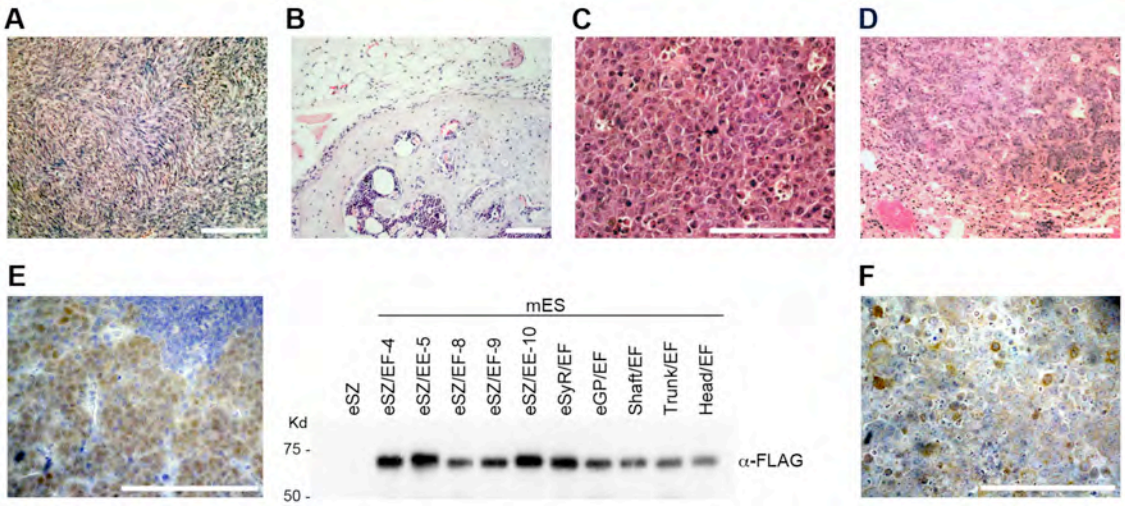
Miwa Tanaka, Yukari Yamazaki, Yohei Kanno, Katsuhide Igarashi, Ken-ichi Aisaki, Jun Kanno, and Takuro Nakamura



Supplemental Figure 1

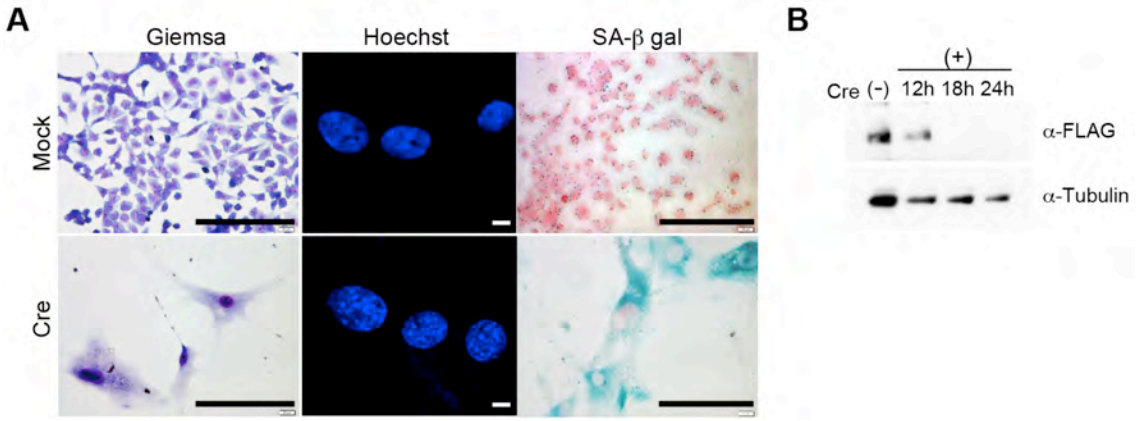
Retrovirus-mediated introduction and expression of fusion genes in mouse embryonic cells. **(A)** Retroviral transduction of *EWS-FLI1* into eSyR, eSZ, eGP, shaft, trunk and head cells is indicated by GFP-positive fractions. *EWS-CHOP* or *SYT-SSX1* transduction in eSZ cells is also shown. **(B)** Expression of EWS-FLI1, EWS-CHOP and SYT-SSX1. The same cell fractions as above were permeabilized, stained with TRITC-labeled anti-FLAG, and analyzed by flow cytometry. (C) Detection of FLAG-tagged EWS-FLI1 in eSyR, eSZ and eGP cells by immunofluorescence using TRITC-labeled anti-FLAG and nuclear counterstaining with DAPI. Scale bar: 20 μm. (D) Expression of EWS-FLI1 in Pthlh^{+} and Pthlh^{neg} fractions of

eSZ cells analyzed by flow cytometry same as (B).



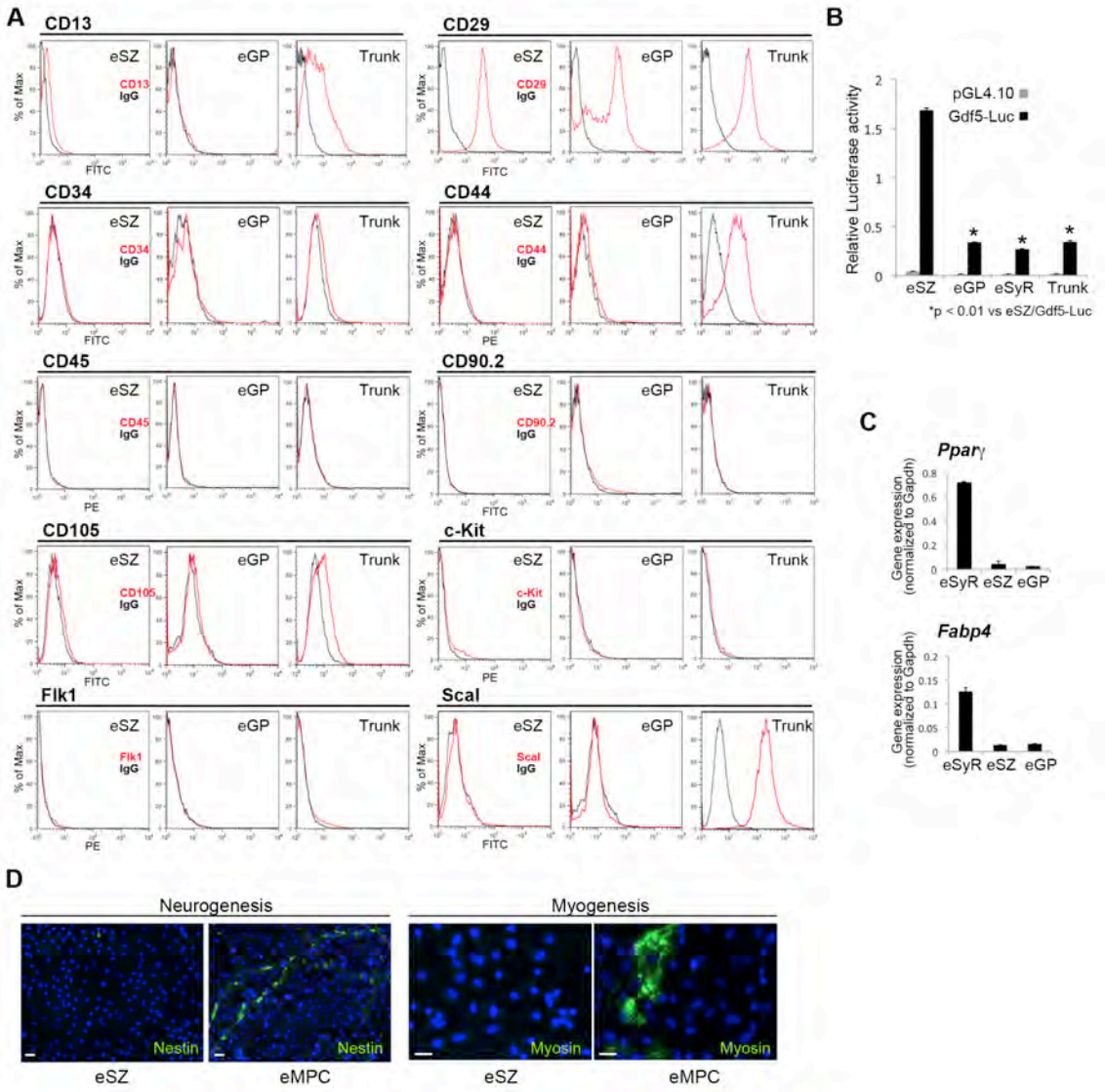
Supplemental Figure 2

Characterization of mouse Ewing's sarcoma. (A) Undifferentiated fibrosarcoma-like tumor originated from embryonic mesenchymal cells transduced with *EWS-FLI1*. **(B)** Non-neoplastic bone and cartilage developed by transplanting eSZ cells introduced with an empty vector. H&E staining. **(C)** Secondary transplanted mouse Ewing's sarcoma. H&E staining. **(D)** Lung metastasis of mouse Ewing's sarcoma injected via tail vein. H&E staining. **(E)** Expression of EWS-FLI1 is detected by immunostaining (left) and immunoblot (right) using anti-FLAG. mES: mouse Ewing's sarcoma derived from the indicated cell types, EF: EWS-FLI1, EE: EWS-ERG. **(F)** Cd99 immunostaining. Tumor cells are positive in parts. Scale bar: 100 μm.



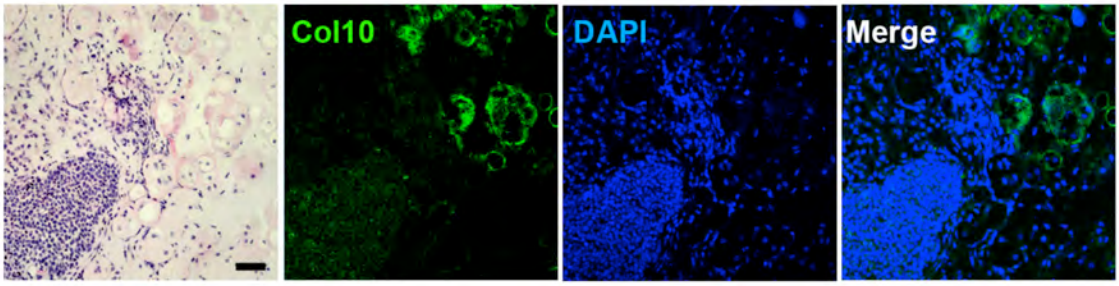
Supplemental Figure 3

Cre/*loxP*-mediated knockout of the *EWS-FLI1* transgene. (A) *EWS-FLI1* knockout induced senescence-like morphologies (left). Senescence-associated heterochromatin foci (center). Senescence-associated β-galactosidase expression (right). (B) Cre-loxP-mediated knock-out of EWS-FLI1 is confirmed at different time points indicated by immunoblotting using anti-FLAG. Scale bar: 100 μm.



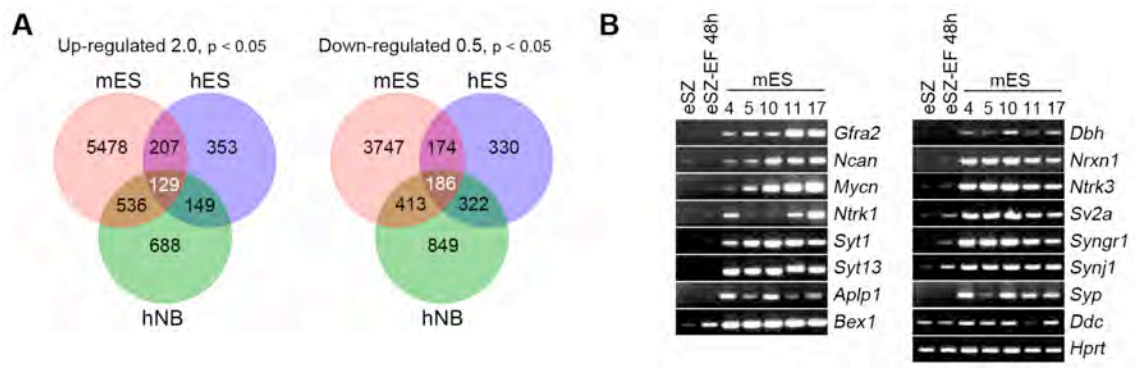
Supplemental Figure 4

Characterization of eSZ cells and murine Ewing's sarcoma. **(A)** Expression of MSC markers, CD13, CD29, CD34, CD44, CD45, CD90.2, CD105, c-Kit, Flk1 and Sca1 in eSZ, eGP and eMPC. Red lines indicate specific antibodies and blue lines indicate isotype-matched IgG control. **(B)** *Gdf5* promoter activities in eSZ, eGP, eSyR and eMPC were assessed by the luciferase assay. Relative luciferase activities are indicated. **(C)** Quantitative RT-PCR for *Pparγ* and *Fabp4* genes related to adipogenesis. **(D)** Neurogenic and myogenic differentiation abilities were compared between eSZ and eMPC by nestin or myosin expression, respectively. Scale bar: 20 μm.



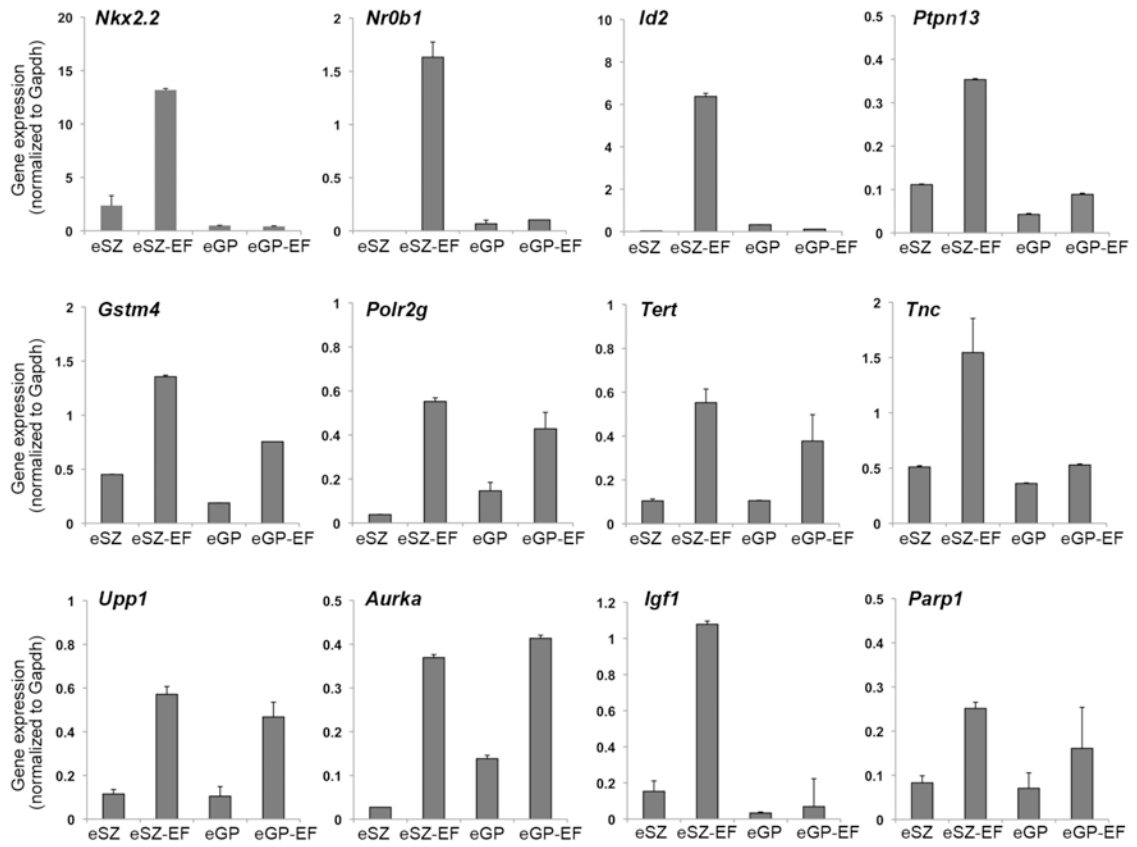
Supplemental Figure 5

Differentiated cells showing chondrocytic differentiation in a peripheral area as demonstrated by collagen 10 staining. Scale bar: 50 μm.



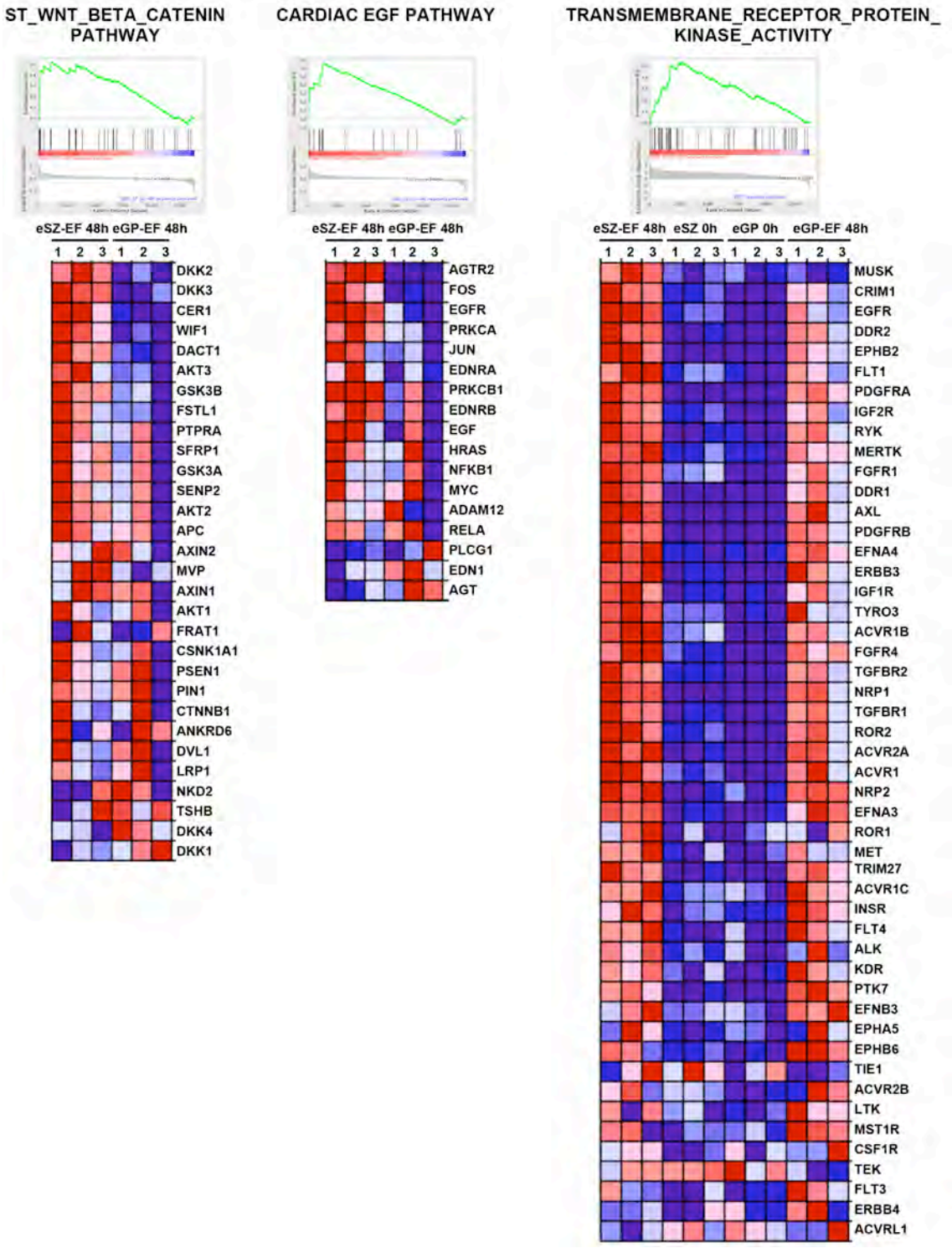
Supplemental Figure 6

Gene expression profiles of Ewing's sarcoma. **(A)** Venn diagrams for common upregulated or downregulated genes among murine and human Ewing's sarcomas, and human neuroblastoma. **(B)** Expression of neural-related genes in murine Ewing's sarcoma was confirmed by RT-PCR. Note that expression of *Gfra2, Ncan, Mycn, Ntrk1, Syt1, Syt13, Aplp1, Bex1, Dbh, Nrxn1, Ntrk3, Sv2a, Syngr1*, *Synj1, Syp* and *Ddc* was also upregulated in eSZ cells by EWS-FLI1 introduction. *Hprt* was used to confirm the quality and quantity of RNA.



Supplemental Figure 7

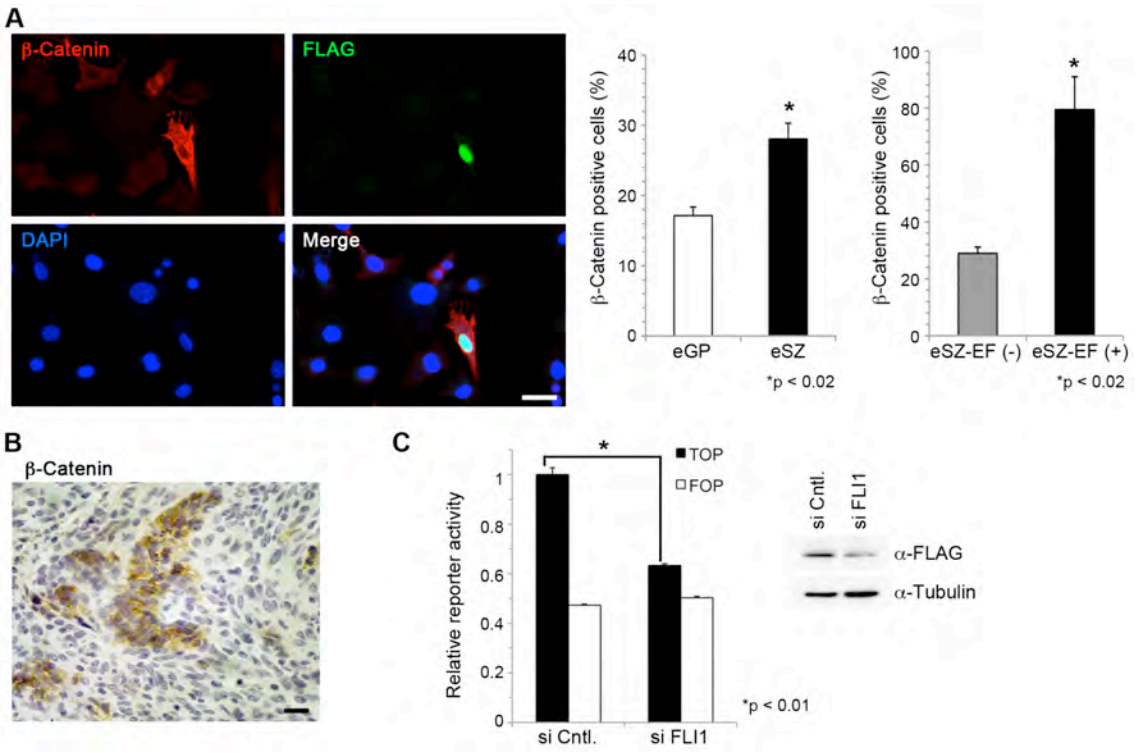
Modulation of gene expression following introduction of *EWS-FLI1*. Quantitative RT-PCR for 12 representative genes in eSZ and eGP cells with or without *EWS-FLI1*.



Supplemental Figure 8

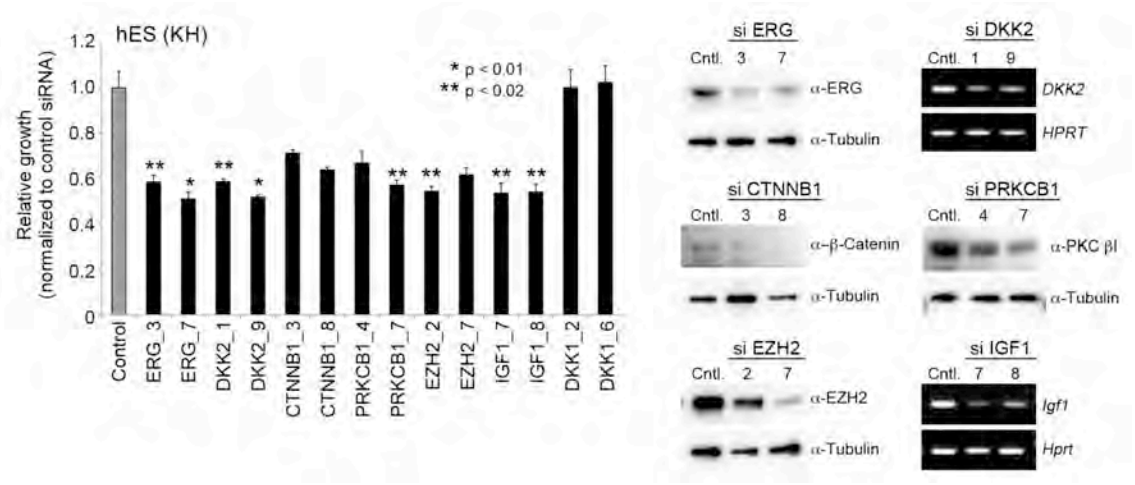
Gene set enrichment analysis. Gene sets in eSZ/EWS-FLI1 vs eGP/EWS-FLI1 (left and center), or eSZ/EWS-FLI1 vs eSZ, eGP and eGP/EWS-FLI1 (right) were compared by GSEA, showing enrichment of

Wnt/β-catenin, EGF or receptor protein kinase activity gene sets.



Supplemental Figure 9

Upregulation of β-catenin expression by EWS-FLI1. **(A)** β-catenin expression is enriched by EWS-FLI1 introduction in eSZ cells. Immunofluorescence staining for EWS-FLI1 (FLAG, green) and β-catenin (red) (left). Frequencies of β-catenin-positive cells in eGP and eSZ cells (center) and in eSZ cells with or without EWS-FLI1 (right). The experiment was performed in triplicate and average numbers of β-catenin-positive cells ± SD in 5,000 or 50 cells are plotted in eGP/eSZ or eSZ-EF (-)/(+), respectively. Scale bar: 20 μm. **(B)** β-catenin expression in mouse Ewing's sarcoma in the invasive area. β-catenin-positive cells are shown as DAB staining. Scale bar: 20 μm. **(C)** Decreased transcriptional activity of β-catenin by *EWS-FLI1* knockdown. Luciferase activities were measured with the TOPFLASH reporter (left). Knock-down of EWS-FLI1 is confirmed by immunoblotting using anti-FLAG (right).



Supplemental Figure 10

Growth suppression of human Ewing's sarcoma cells with *EWS-ERG* by gene silencing. Inhibition of cell proliferation by knockdown of *ERG1* and genes of important pathways. Relative growth of tumor cells 48 hr after siRNA treatment was calculated by comparing treated cell number to cells subjected to control siRNA. The mean and SEM in three independent experiments are shown (left). *Dkk1* was tested as a negative control. Gene knockdown was confirmed by immunoblotting (ERG, CTNNB1, EZH2 and PRKCB1) or RT-PCR (*DKK2* and *IGF1*) (right).

Supplemental Table 1

Details of transplantation and metastasis experiments.

Transplantation

mES#	# of recipients	incidence of tumor (%)	average latency (week)
1	1	100	2
2	1	100	4
3	1	100	3
17	1	100	3
18	1	100	3
19	1	100	3
*20	3	100	2.67
36	1	100	3
L1	6	100	2.33
L2	11	100	1.27

***Tertiary transplantation was confirmed in #20.**

Metastasis by tail vein injection

mES#	# of recipients	incidence of metastasis (%)	average latency (week)
17	1	0	-
19	2	0	-
20	1	100	12
21	1	0	-
22	1	0	-
24	1	0	-
25	3	0	-
26	2	100	8.5
29	2	100	9

Supplemental Excel File 1.

The list of retroviral integration sites in murine Ewing's sarcomas

The retroviral integration sites were identified by inverse-PCR using specific primers for pMYs-EWS-FLI1-IRES-GFP.

Supplemental Table 2. The number of genes differentially expressed in eSZ and eGP cells with/without *EWS-FLI1*.

Samples	Mean fold change of geometric[a]	
	>2.0	<0.5
eSZ vs. eGP	1107	482
eSZ/*EWS-FLI1* vs. eSZ	14016	191
eSZ/*EWS-FLI1* vs. eGP/*EWS-FLI1*	665	370

[a]Mean-fold change was compared between indicated groups. Statistical significance was tested by Student's t-test assuming unequal variances (P<0.05).

Supplemental Excel File 2. The list of genes differentially expressed between eSZ and eGP

Gene expression data were obtained by hybridizing 3 cell samples from each experimental group. Adjusted P-values below 0.05 were considered significant.

Supplemental Excel File 3.

The list of common upregulated genes between murine and human Ewing's sarcoma, and human neuroblastoma.

The microarray data from ten murine Ewing's sarcoma samples were compared with human microarray datasets. Selection of common upregulated genes were achieved based on the data of 12,340 probes were selected by one-way ANOVA (P<0.05) analysis.

Supplemental Excel File 4.

The list of common downregulated genes between murine and human Ewing's sarcoma, and human neuroblastoma.

The microarray data from ten murine Ewing's sarcoma samples were compared with human microarray datasets. Selection of common downregulated genes were achieved based on the data of 12,340 probes were selected by one-way ANOVA (P<0.05) analysis.

Supplemental Excel File 5.
The list of genes differentially expressed between eSZ/EWS-FLI1 and eSZ

Gene expression data were obtained by hybridizing 3 cell samples from each experimental group. Adjusted p-values below 0.05 were considered significant.

Supplemental Excel File 6, a list of primer sequences used in the study.

Supplemental Excel File 7.
The list of siRNAs.