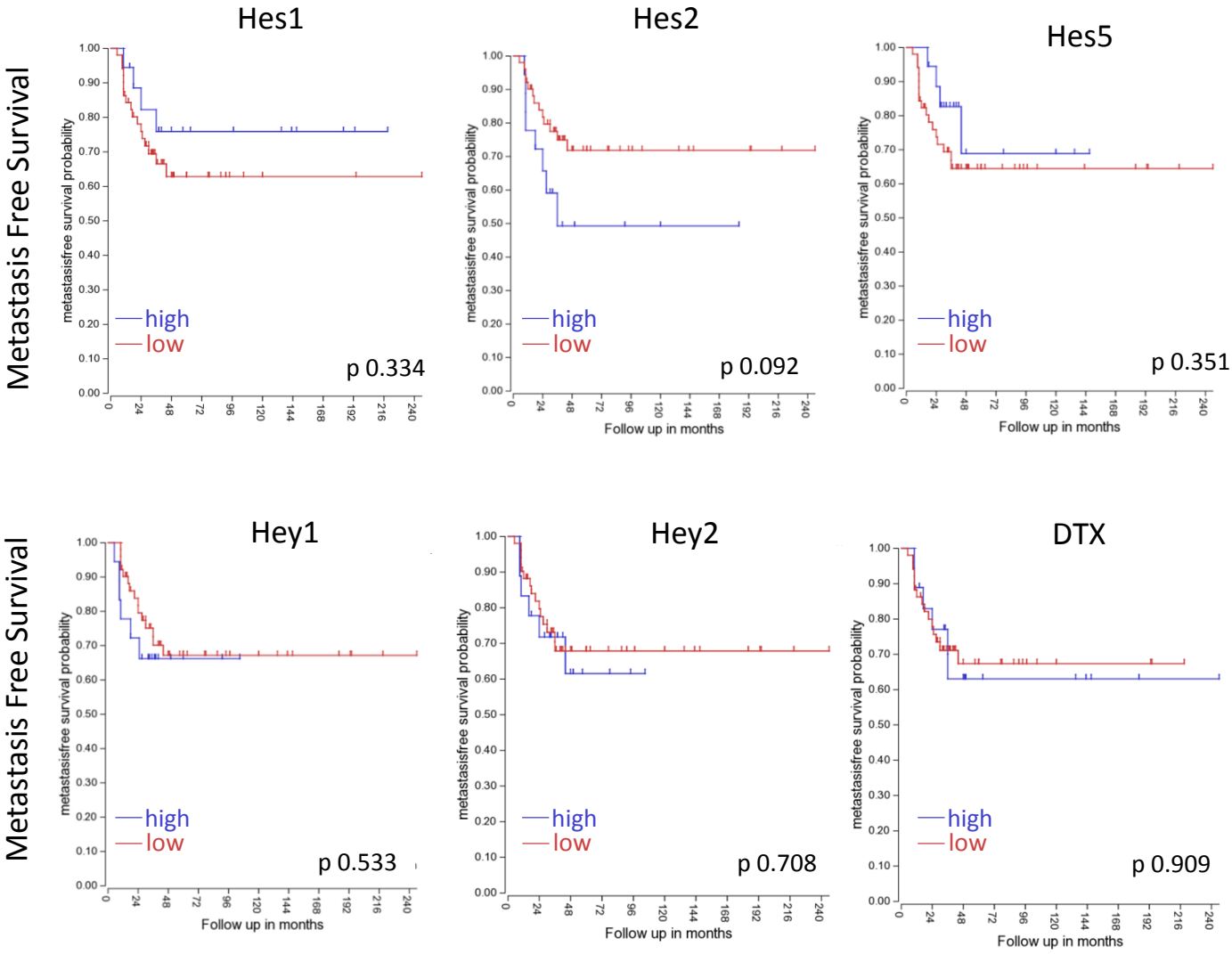
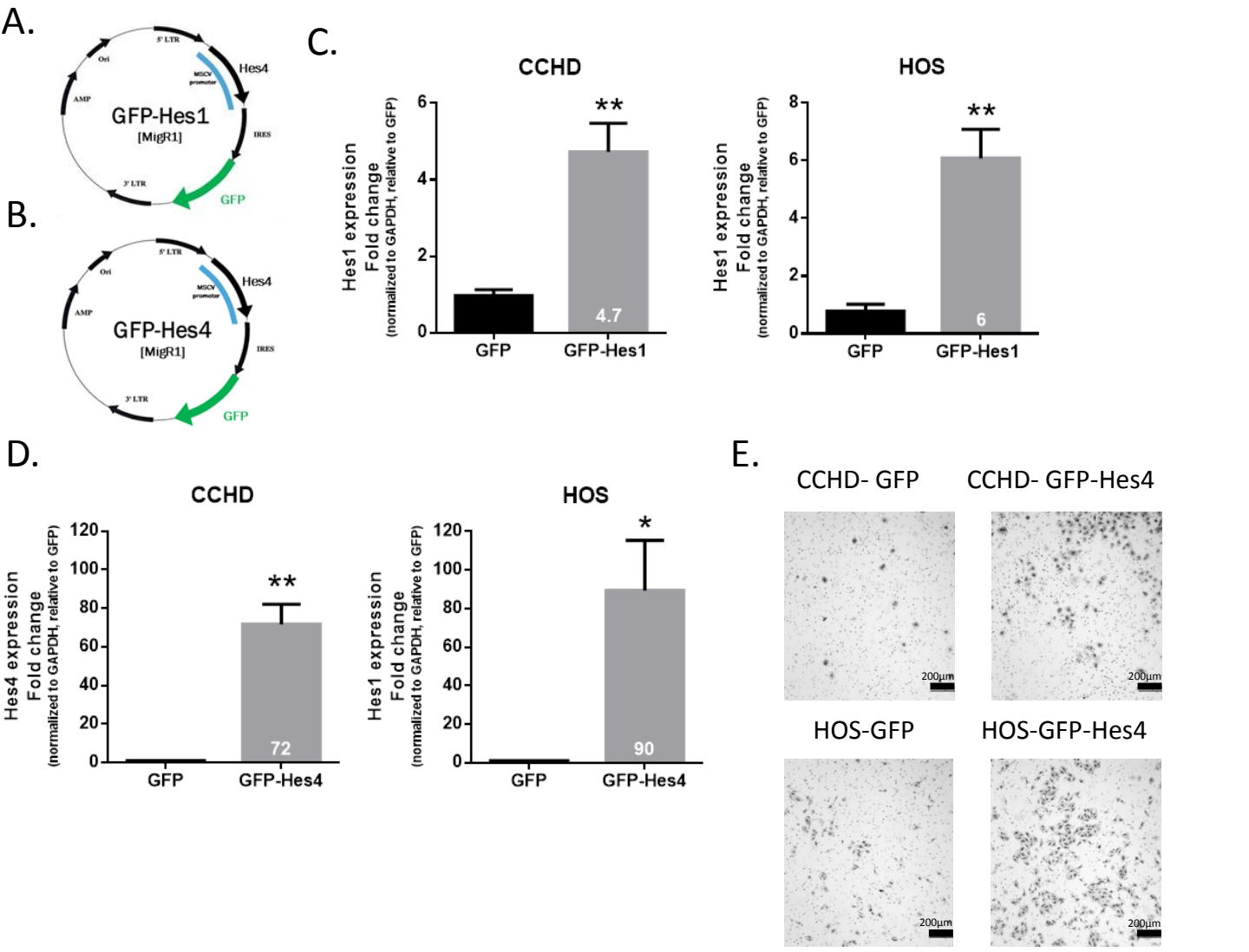
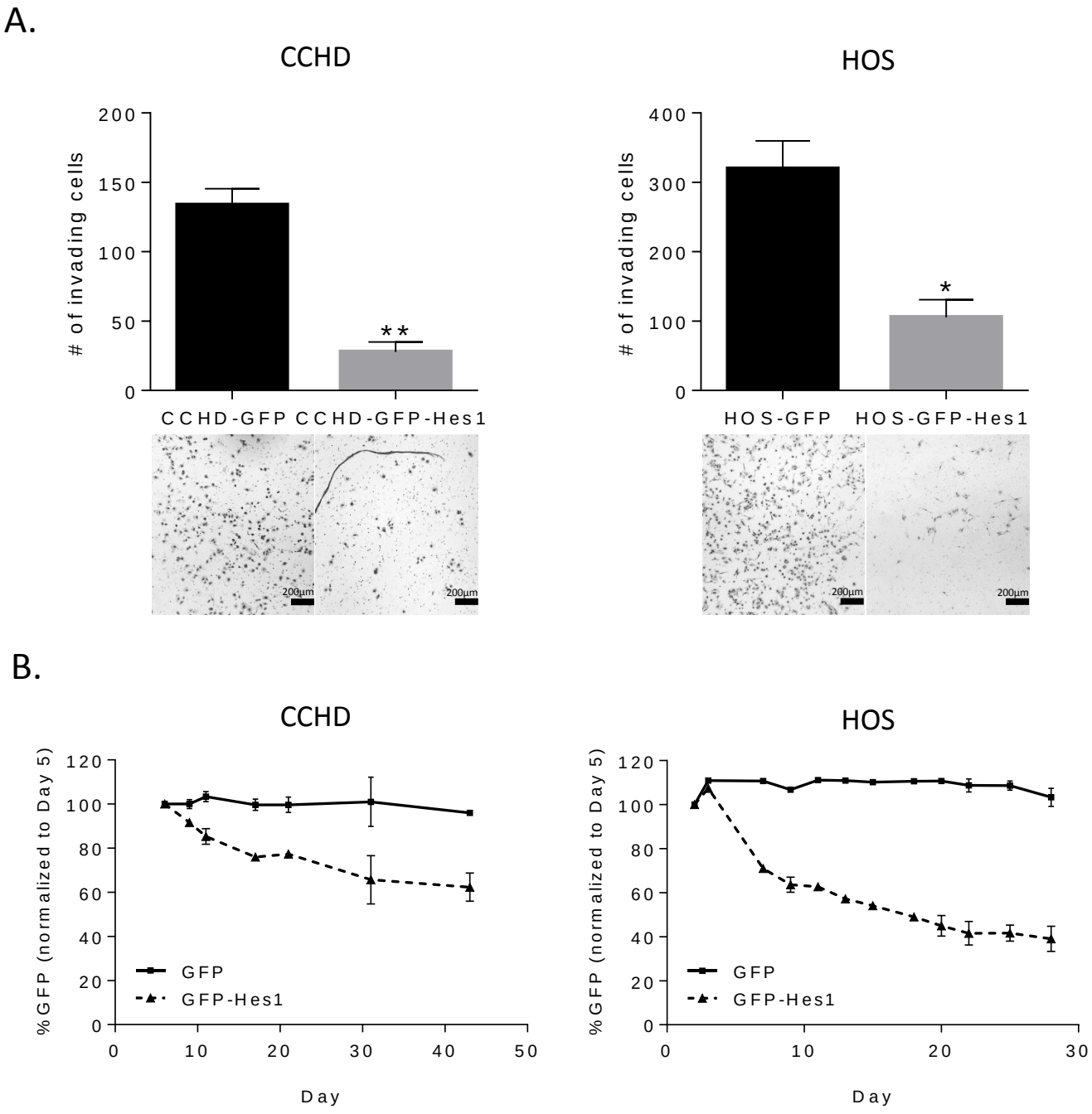
Supplemental Data



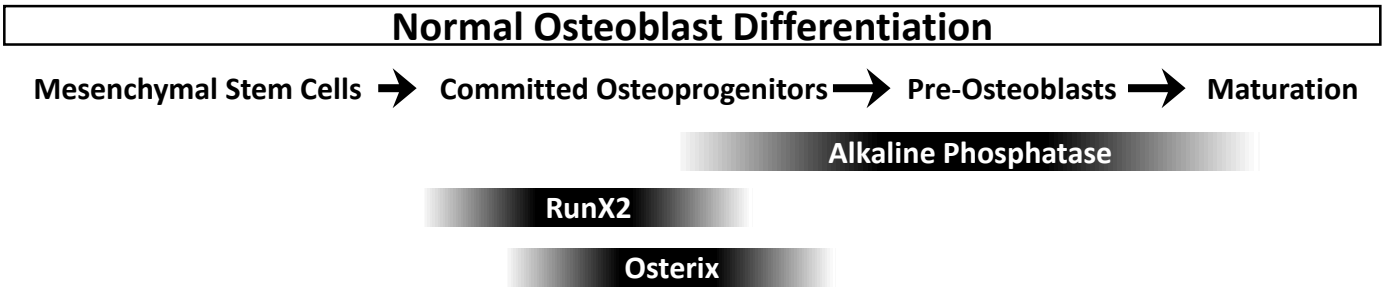
Supplementary Figure S1. Correlation of OS patient outcome and expression of Notch downstream targets other than Hes4. The R2: Genomics Analysis and Visualization Platform was used to generate Kaplan-Meier metastasis-free and overall survival curves using the Mixed Osteosarcoma - Kuijjer - 127 - vst - ilmnhwg6v2 data set. Genome-wide gene expression analysis was performed using 84 pretreatment high-grade diagnostic OS biopsy samples. Two different sets of control samples were used for comparison: osteoblasts (n = 3) and mesenchymal stem cells (n = 12; GEO accession number GSE28974). Hes1, Hes2, Hes5, Hey1, Hey2, and DTX were classified according to high versus low expression. No significant differences were seen in any of the groups.



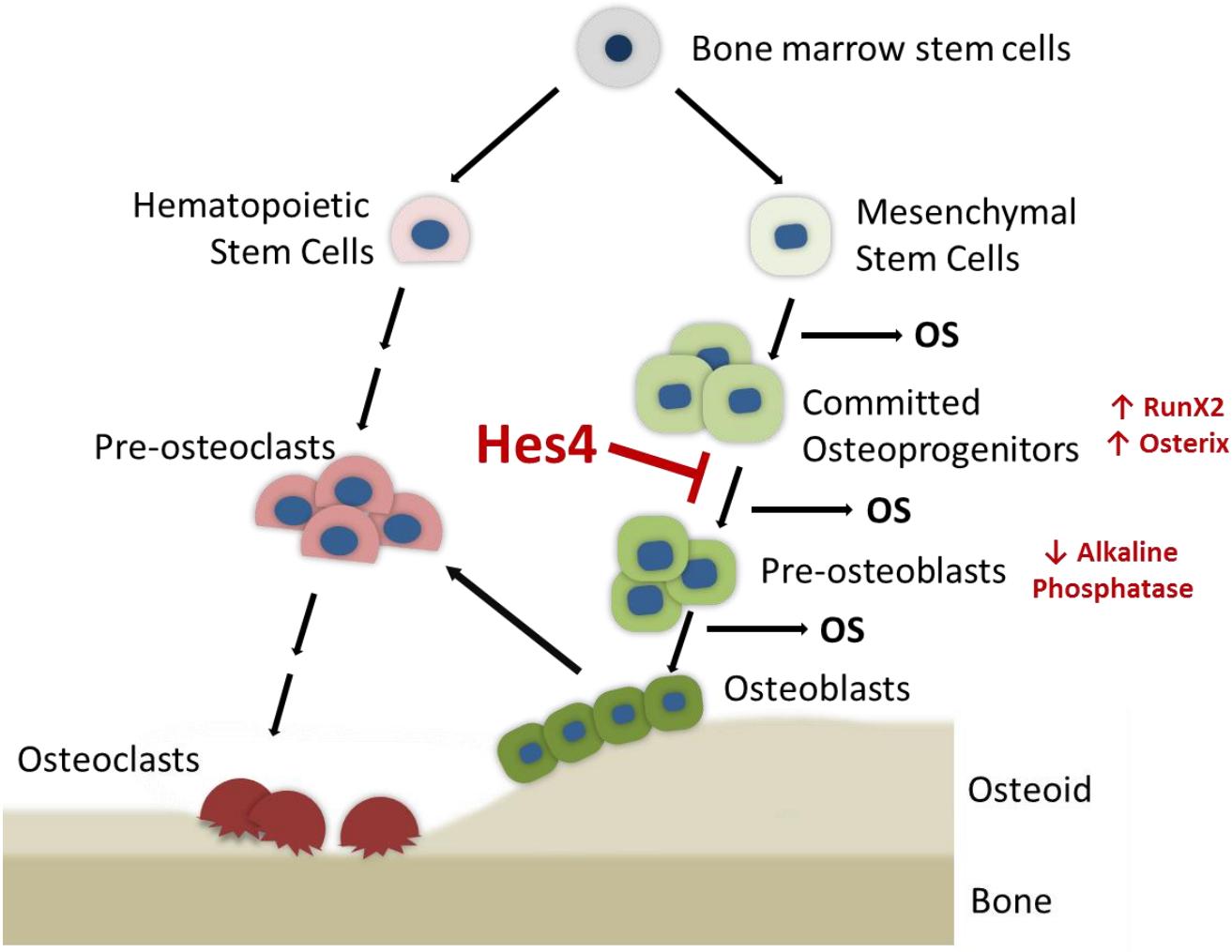
Supplementary Figure S2. Hes1 and Hes4 overexpression in CCHD and HOS cells. A and B, schematic representations of Hes1 (A) and Hes4 (B) overexpression vector maps depicting the orientation of GFP and Hes1 or Hes4 within the retroviral MigR1 backbone. The expression of Hes1 and Hes4 is controlled by a constitutively active 5' LTR promoter. The presence of an IRES causes production of Hes1 or Hes4 and GFP as two separate proteins, not a fusion protein. C, Transduction of Hes1 results in a significant increase of Hes1 RNA expression. cDNA was prepared from RNA harvested from HOS and CCHD cells after transduction with GFP or GFP-Hes1. RT-qPCR was performed to measure the levels of Hes1 expression normalized according to GAPDH expression relative to that in GFP-transduced control cells. (D) Transduction of Hes4 results in a significant increase of Hes1 or Hes4 RNA expression, respectively. cDNA was prepared from RNA harvested from HOS and CCHD cells after transduction with GFP or GFP-Hes4. RT-qPCR was performed to measure the levels of Hes4 expression normalized according to GAPDH expression relative to that in GFP-transduced control cells. $*P \leq 0.05$; $**P \leq 0.01$. Bars, mean ± SEM (n = 3). (E) Representative images of the bottom well of the migration assay using CCHD-GFP and CCHD-GFP-Hes4 and HOS-GFP and HOS-GFP-Hes4, respectively.



Supplementary Figure S3. Hes1 overexpression in CCHD and HOS cells decreases OS invasion and proliferation. (A) Hes1 overexpression decreases invasion in CCHD and HOS cells. CCHD and HOS cells were transduced with GFP or GFP-Hes1 and sorted according to GFP positivity. Their Invasiveness was measured using a 24-well BioCoat Matrigel invasion chamber with an 8-µm pore size. A medium with 10% fetal bovine serum was used in the bottom well of the chamber as a chemoattractant. At 24 (HOS) or 48 (CCHD) hours, migrated cells were counted. The graph shows the mean number of migrated cells per field (± SEM; $n = 3$). *$P \leq 0.05$; **$P < 0.01$. (B) Hes1 overexpression decreases proliferation in CCHD and HOS cells. The percentages of GFP-positive CCHD and HOS cells over time after stable retroviral transduction of GFP or GFP-Hes1 (normalized to day 5 after transduction) were quantified at various time points as described in Materials and Methods and expressed as the mean cell number ± SEM ($n = 3$).



Supplementary Figure S4. Schematic depicting key transcription factors involved in normal osteoblast differentiation. Differentiation stage is defined by the presence or absence of specific transcription factors and can be divided into four main stages: pluripotency, osteogenic commitment, preosteoblast/early osteoblast, and maturation.



Supplemental Figure S5. Schematic depicting the highly regulated balance of osteoblasts and osteoclasts, and the role of Hes4 in the inhibition of osteogenic differentiation in OS. Bone remodeling relies on both osteoclastic and osteoblastic activity. The formation of osteoclasts and osteoblasts is highly regulated by a multistep differentiation process. Osteoclasts originate from hematopoietic stem cells while osteoblasts originate from mesenchymal stem cells. There is cross talk between osteoblasts and pre-osteoclasts (via IL-1α/RANKL/RANK signaling). Osteosarcoma is thought to arise from the disruption of osteogenic differentiation, and can occur at any point within the differentiation pathway resulting in a heterogeneous mix of OS which represents multiple maturation states. Defects at early stages within the osteogenic differentiation pathway leads to the development of more aggressive and less differentiated OS. Hes4 blocks the osteogenic differentiation pathway by preventing the maturation of pre-osteoblasts by increasing RunX2 and osterix, and decreasing alkaline phosphatase. The Hes4 mediated block of differentiation results in large primary tumors and significantly more metastases in vivo, and correlates with decreased metastasis free and overall survival in high grade OS patients.