Supplemental Materials and Methods

Transfection of MOS-J cells:

One million MOS-J cells were transfected with a NucleofectorII (Lonza, Allendale, NJ USA) using the manufacturer's settings for Ewing sarcoma cells. Stable lines were generated by Blasticidin selection. The transfected MOS-J cells were also transduced with a lentiviral construct encoding dsRed-mito driven by the cytomegalovirus promoter (pDsRed2-Mito, BD Biosciences, San Jose, CA, USA) to fluorescently label mitochondria. To achieve >99% purity, cells were enriched using a MoFlo XDP cell sorter (Beckman Coulter, Miami, FL, USA). In some experiments, parental MOS-J cells received plasmid constructs constitutively expressing c-Jun[S1] (pMEIG3-c-Jun; plasmid 40348, Addgene Cambridge, MA, USA) or ALDH (pcDNA-HA-ADH; Addgene plasmid 11610 from the S. Johnson group) by nucleofection.

Osteogenic differentiation assays:

To test the effect of Dkk-1 on *in vitro* osteogenic differentiation capabilities, confluent monolayers of MOS-J cells were incubated in base medium supplemented with 100 nM dexamethasone, 50 $\mu g \cdot mL^{-1}$ ascorbic acid, and 5 mM β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO, USA) for 15 days with changes every 2 days. Alkaline phosphatase levels were measured after 8 days as described previously[2] by a kinetic assay based on the conversion of p-nitrophenol phosphate to nitrophenolate using a plate reader (FluoStar, BMG Labtech, Cary, NC, USA). Mineralization was confirmed by Alizarin Red staining (Sigma).[S2-3]

5-ethynyl-2´-deoxyuridine (EdU) incorporation assay:

MOSJ-Dkk1 and control cells were seeded at 3,000 cells/well of a 96 well plate in quadruplicate and cultured over 12 days. Every other day, cells were pulsed with 10μM EdU for 4h (Click-iT EdU Microplate Assay; Molecular Probes, Eugene, OR, USA). Cells were fixed, processed according to the manufacturer's protocol and analyzed on a plate reader.

ELISAs:

A Dkk-1 duo-set was obtained from R&D Systems (Minneapolis, MN, USA) and utilized in accordance with the manufacturer's directions.

Cell cycle analysis:

300,000 cells were washed in PBS, fixed for 15 min with DNA Prep LPR and stained for at least 2 h with DNA Prep Stain (Coulter, Miami, FL, USA). DNA content profiles were generated by flow cytometry (FC500, Beckman Coulter). At least 5,000 single cell cycle events were acquired and analyzed with modeling software (MultiCycleAV, Phoenix Flow Systems, San Diego, CA, USA).

Annexin V staining:

300,000 cells were washed in PBS, incubated in annexin-binding buffer containing FITC- or PE-labeled AnnexinV (Sigma, Becton Dickinson) and 1 $\mu g \cdot mL^{-1}$ 7-aminoactinomycin D (7-AAD, Invitrogen). After incubation for 15 min, cells were analyzed on a Beckman Coulter FC500 flow cytometer. At least 30,000 events were acquired and analyzed using CPR software (Beckman Coulter).

Western blotting:

MOS-J cells were analyzed by western blotting as described previously.[S4] In brief, cells were lysed in PBS containing 1% Triton X-100 (Sigma) and a protease/phosphatase inhibitor cocktail (Complete and PhosSTOP, Roche Diagnostics, Indianapolis, IN, USA). The insoluble fraction containing membrane bound β-catenin was recovered by centrifugation and the supernatant was separated by SDS PAGE and blotted following standard protocols. Membranes were probed with 1 in 1,000 dilutions of rabbit anti-β-catenin antibody (Abcam, Cambridge, MA, USA), rabbit-anti-JNK, rabbit-anti-phospho-JNK, rabbit-anti-p38 kinase, rabbit-anti-phospho-p38 kinase, rabbit-anti-MAP kinase-kinase-7 (MKK7), rabbit-anti-phospho-MKK7, rabbit-anti-MAP kinase-kinase-4 (MKK4), rabbit-anti-phospho-MKK4 (Cell Signaling Technology, Danvers, MA, USA), and mouse anti-GAPDH (MAB374, Chemicon EMD Millipore, Billerica, MA, USA). Primary antibody binding was probed using peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (dilution 1:1,000, Sigma). Blots were developed using chemiluminescence.

Immunocytochemistry:

Immunocytochemistry for β-catenin was performed as previously described[S5] using a fluorescein isothiocyanate conjugated monoclonal antibody to β-catenin (clone 14, Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ) at a concentration of 1 in 400. Controls were stained with Alexa-fluor 488 conjugated isotype control antibody (Invitrogen). Nuclei were stained with mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Images were captured on a Nikon Eclipse 80i microscope fitted with a Retiga 2000R camera using NIS elements AR (ver 3.1, Nikon Instruments, Melville, NY, USA).

RNA extraction

Total RNA was extracted from cell pellets using the HighPure RNA Isolation kit (Roche) following the manufacturer's protocol. RNA quality was assessed by spectrophotometry and PCR for murine GAPDH after reverse transcription using the SuperScript III kit (Invitrogen).

Microarray:

500 ng of each RNA sample were labeled using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. A total of 15 µg labeled RNA was fragmented and hybridized onto mouse arrays (MG-430 2.0, Affymetrix). After normalization, gene level analyses and comparisons were done using the Partek Genomics Suite software (version 6.4, Partek, St. Louis, MO, USA). For gene-ontology and database comparisons, analysis of data was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7).[S6] Microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE43112 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43112).

Conventional end-point and qRT-PCR:

To confirm microarray data, the expression of several candidate genes were measured by quantitative real time-PCR (qRTPCR) using FastSybr green chemistry (Applied Biosystems, Carlsbad, CA, USA) or the WNT Signaling Pathway RT² Profiler PCR Array (Qiagen, Valencia, CA, USA) on a CFX96 Detection System (Bio-Rad, Berkeley, CA, USA). Expression data were calculated using the $2^{-\Delta\Delta CT}$ method.[S7] For conventional PCR, cDNAs were amplified in 25µl reactions consisting of PCR SuperMix (Invitrogen) and 50 pmoles of the respective primer pairs on a C1000 thermocycler (Bio-Rad) and visualized by agarose gel electrophoresis. cDNA from

murine E12.5 embryos was used as positive control. Primer sequences are given in the table below:

Target	Sequence	Reference
Murine Aldh1a1	FOR 5′-GACAGGCTTTCCAGATTGGCTC-3′ REV 5′-AAGACTTTCCCACCATTGAGTGC-3′	S8
Murine Aldh1a7	FOR 5′-GCTGTCCCTGTCCAATGCCCATTTG-3′ REV 5′-GGTGACTGTATGAGATGTACAGCCAC-3′	S8
Murine Axin2	FOR 5′-GAGTAGCGCCGTGTTAGTGACT-3′ REV 5′-CCAGGAAAGTCCGGAAGAGGTATG-3′	S9
Murine Gapdh	FOR 5′-CATGGCCTTCCGTGTTCCTA-3′ REV 5′-GCGGCACGTCAGATCCA-3′	RTPrimerDB ID_473 S10
Human ALDH1A1	FOR 5′-CGCAAGACAGGCTTTTCAG-3′ REV 5′-TGTATAATAGTCGCCCCCTCTC-3′	S11
Murine Ror1	FOR 5'-GCTGCGGATTAGAAACCTTG-3' REV 5'-TACGGCTGACAGAATCCATC-3'	S12
Murine Ror2	FOR 5'-TCCTTCTGCCACTTCGTCTT-3' REV 5'-TTGTAGCACTGGTGGTAGCG-3'	S13
Murine Rac1	FOR 5'-GTGCTCAGCTCTCACACAGC-3' REV 5'-CAGCAGGCATTTTCTCTTCC-3'	S14
Murine Rac2	FOR 5'-ATGTCCGTGCCAAGTGGT-3' REV 5'-CTTCTGCTGTCGTGTGGG-3'	S15
Murine Daam1	FOR 5'-GTTCTCTGCCTACCAAAGACAGC-3' REV 5'-CCGACCATCAATCACTGACAGC-3'	Origene primer MP220706
Murine Rhoa	FOR 5'-CTTCAGCAAGGACCAGTTCCCA-3' REV 5'-GGCGGTCATAATCTTCCTGTCC-3'	Origene primer MP212453
Human GAPDH	FOR 5′-CTCTCTGCTCCTCCTGTTCGAC-3′ REV 5′-TGAGCGATGTGGCTCGGCT-3′	S16

RNAi knockdown of Dkk-1:

RNAi knockdown of Dkk-1 was performed as recommended by the manufacturer (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Briefly, 250,000 MOSJ-Dkk1 cells were transfected with siRNA specific to Dkk-1 in a 54 cm^2 tissue culture plate. The transfection mixture was incubated with the cells in the presence of low-serum media (Opti-MEM,

Invitrogen) for 15 h, followed by addition of an equal volume of standard culture media containing 20% FBS. Cultures receiving scrambled RNAi served as controls. After 5 days, RNA was extracted and analyzed.

Inhibition of cWnt, GSK3β, RhoA, ALDH and AP-1 signal transduction:

After 3-5 days of GSK3β inhibitor treatment, RNA was extracted and analyzed as above. To inhibit AP-1 mediated signaling, MOSJ-Dkk1 cells received 10μM nordihydroguaiaretic acid (NDGA) or curcumin in dimethyl sulphoxide (DMSO). Controls received vehicle. Cultures were incubated with the agents for 5 days prior to assays.[S17] To inhibit ALDH activity under conditions of metabolic stress, 0.15-15 μM DEAB[S18] or 0.05-5 mM chloramphenicol (Sigma)[S19-20] was added to confluent cultures of MOS-J cells with supplementation of drug every 3.5 days for up to 21 days. Vehicle controls were 1:1000 original volume DMSO or ethanol respectively. Cells were enumerated using a fluorescent DNA detection assay as previously described.[S2] To inhibit β-catenin activity, the antagonist CCT036477 (EMD Millipore)[S21] was utilized at 1, 5 and 10 μM. For qRT-PCR studies, the most protective concentration (5 μM) was employed. To inhibit tankyrase, tankyrase inhibitor III (TNKiIII) (EMD Millipore) was utilized (3-(4-Methoxyphenyl)-5-((4-(4-methoxyphenyl)-5-methyl-4H-1,2,4-triazol-3-ylthio)methyl)-1,2,4-oxadiazole) at 0.1, 1.0 and 10.0 μM.[S22] For qRT-PCR studies, the most protective concentration (10 μM) was employed. To show/establish the link between RhoA-mediated phosphorylation of Jnk and Aldh expression levels, MOSJ-Dkk1 cells were incubated for up to 10 hours with 1 μg mL^{-1} of Rho inhibitor I (CT04; Cytoskeleton Inc., Denver, CO, USA)[S23] or vehicle and analyzed by qRT-PCR and immunoblotting as above.

Micro-CT (μCT):

For histomorphometry, scans were performed at 12 μm pixel resolution and axial reconstructions were generated (NRecon software version 1.6.4, Skyscan, Bruker-microCT, Kontich, Belgium). Regions of interest (ROIs) corresponding to the tumor bone interface were identified and histomorphometric parameters of adjacent bone were measured as before (CTAn software version 1.12.0.0+, Skyscan).[S24] MOSJ-Dkk1 tumors were observed to have a maximal destructive effect on the fibulae. Therefore, ROIs of 4 mm^3 over the central 100 axial cross-sections of fibulae (corresponding to the central one third of the length of the fibulae) were selected and thresholded to a density that corresponded to the unaffected contralateral side. Bone volume was then calculated and expressed as a proportion of the region of interest. To visualize soft tissue, legs were contrast-stained with 50% Lugol's iodine/potassium iodide solution (adapted after ref. [S25]) for up to 11 days and re-scanned in their entirety at 30 μm resolution. Axial reconstructions indicated that the margins of the MOSJ-Dkk1 tumors were not well defined and this prohibited direct volumetric measurements of the tumor. Instead, axial reconstructions were individually inspected for presence of tumor tissue and proximal and distal limits were set so as to define a longitudinal ROI that included the entire tumor. The entire tissue volume was calculated between these limits and compared with the unaffected contralateral limb in each case. Using this method, the size of the tumor and associated stroma could be estimated based on the baseline volume of an anatomically equivalent ROI on the contralateral limb.

Histology:

Scanned legs were decalcified in 0.5 M EDTA (Sigma), embedded in paraffin (Richard-Allan Scientific, Kalamazoo, MI, USA) and cut into 8 μm-thick sections. Sections were stained with hematoxylin and eosin or Masson's trichrome (American Mastertech Scientific, Lodi, CA,

USA). Cellular subtype and morphological characteristics of the tumor were classified by a blinded clinical osteopathologist.

Supplemental References.

S1. Wang ZY, Sato H, Kusam S, Sehra S, Toney LM, Dent AL. Regulation of IL-10 gene expression in Th2 cells by Jun proteins. *J Immunol* 2005 Feb; **174**(4)**:** 2098-2105.

S2. Krause U, Seckinger A, Gregory CA. Assays of osteogenic differentiation by cultured human mesenchymal stem cells. *Methods Mol Biol* 2011; **698:** 215-230.

S3. Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* 2004 Jun 1; **329**(1)**:** 77-84.

S4. Gregory CA, Perry AS, Reyes E, Conley A, Gunn WG, Prockop DJ. Dkk-1-derived synthetic peptides and lithium chloride for the control and recovery of adult stem cells from bone marrow. *J Biol Chem* 2005 Jan 21; **280**(3)**:** 2309-2323.

S5. Krause U, Harris S, Green A, Ylostalo J, Zeitouni S, Lee N, *et al.* Pharmaceutical modulation of canonical Wnt signaling in multipotent stromal cells for improved osteoinductive therapy. *Proc Natl Acad Sci U S A* 2010 Mar 2; **107**(9)**:** 4147-4152.

S6. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**(1)**:** 44-57.

S7. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001 Dec; **25**(4)**:** 402-408.

S8. Levi BP, Yilmaz OH, Duester G, Morrison SJ. Aldehyde dehydrogenase 1a1 is dispensable for stem cell function in the mouse hematopoietic and nervous systems. *Blood* 2009 Feb 19; **113**(8)**:** 1670-1680.

S9. Dao DY, Yang X, Chen D, Zuscik M, O'Keefe RJ. Axin1 and Axin2 are regulated by TGF- and mediate cross-talk between TGF- and Wnt signaling pathways. *Ann N Y Acad Sci* 2007 Nov; **1116:** 82-99.

S10. Pattyn F, Robbrecht P, De Paepe A, Speleman F, Vandesompele J. RTPrimerDB: the real-time PCR primer and probe database, major update 2006. *Nucleic Acids Res* 2006 Jan 1; **34**(Database issue)**:** D684-688.

S11. Visus C, Ito D, Amoscato A, Maciejewska-Franczak M, Abdelsalem A, Dhir R, *et al.* Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. *Cancer Res* 2007 Nov 1; **67**(21)**:** 10538-10545.

S12. Endo M, Doi R, Nishita M, Minami Y. Ror family receptor tyrosine kinases regulate the maintenance of neural progenitor cells in the developing neocortex. *J Cell Sci* 2012 Apr 15; **125**(Pt 8)**:** 2017-2029.

S13. Hatta K, Chen Z, Carter AL, Leno-Duran E, Zhang J, Ruiz-Ruiz C, *et al.* Orphan receptor kinase ROR2 is expressed in the mouse uterus. *Placenta* 2010 Apr; **31**(4)**:** 327-333.

S14. Croke M, Ross FP, Korhonen M, Williams DA, Zou W, Teitelbaum SL. Rac deletion in osteoclasts causes severe osteopetrosis. *J Cell Sci* 2011 Nov 15; **124**(Pt 22)**:** 3811-3821.

S15. Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, *et al.* Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* 1999 Feb; **10**(2)**:** 183-196.

S16. Carraro G, Albertin G, Forneris M, Nussdorfer GG. Similar sequence-free amplification of human glyceraldehyde-3-phosphate dehydrogenase for real time RT-PCR applications. *Mol Cell Probes* 2005 Jun; **19**(3)**:** 181-186.

S17. Ruocco KM, Goncharova EI, Young MR, Colburn NH, McMahon JB, Henrich CJ. A high-throughput cell-based assay to identify specific inhibitors of transcription factor AP-1. *J Biomol Screen* 2007 Feb; **12**(1)**:** 133-139.

S18. Koppaka V, Thompson DC, Chen Y, Ellermann M, Nicolaou KC, Juvonen RO, *et al.* Aldehyde dehydrogenase inhibitors: a comprehensive review of the pharmacology, mechanism of action, substrate specificity, and clinical application. *Pharmacol Rev* 2012 Jul; **64**(3)**:** 520-539.

S19. Allali-Hassani A, Weiner H. Interaction of human aldehyde dehydrogenase with aromatic substrates and ligands. *Chem Biol Interact* 2001 Jan 30; **130-132**(1-3)**:** 125-133.

S20. Kast RE, Belda-Iniesta C. Suppressing glioblastoma stem cell function by aldehyde dehydrogenase inhibition with chloramphenicol or disulfiram as a new treatment adjunct: an hypothesis. *Curr Stem Cell Res Ther* 2009 Dec; **4**(4)**:** 314-317.

S21. Ewan K, Pajak B, Stubbs M, Todd H, Barbeau O, Quevedo C, *et al.* A useful approach to identify novel small-molecule inhibitors of Wnt-dependent transcription. *Cancer Res* 2010 Jul; **70**(14)**:** 5963-5973.

S22. Shultz MD, Kirby CA, Stams T, Chin DN, Blank J, Charlat O, *et al.* [1,2,4]triazol-3-ylsulfanylmethyl)-3-phenyl-[1,2,4]oxadiazoles: antagonists of the Wnt pathway that inhibit tankyrases 1 and 2 via novel adenosine pocket binding. *J Med Chem* 2012 Feb; **55**(3)**:** 1127-1136.

S23. Benink HA, Bement WM. Concentric zones of active RhoA and Cdc42 around single cell wounds. *J Cell Biol* 2005 Jan 31; **168**(3)**:** 429-439.

S24. Gunn WG, Krause U, Lee N, Gregory CA. Pharmaceutical inhibition of glycogen synthetase kinase-3beta reduces multiple myeloma-induced bone disease in a novel murine plasmacytoma xenograft model. *Blood* 2011 Feb 3; **117**(5)**:** 1641-1651.

S25. Metscher BD. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol* 2009; **9:** 11.