Supporting Information

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SI Text

Histology. Knee joints from TOPGAL mice, ages 1, 3, 6, 12, and 18 months were resected from both hind legs, fixed in 10% zinc-buffered formalin (Z-Fix; Anatech) for 1 day, decalcified in TBD-2 (Shandon) for 3 days, followed by paraffin embedding. Serial sections (4 μm of each) were cut, stained with safranin O-fast green, and examined for histopathological changes.

Immunofluorescence Assay. Sections of knee joints from WT mice were prepared as described above. The slides were incubated with rabbit anti-HMGB2 polyclonal antibody (BD PharMingen) at 2 μg/mL and mouse monoclonal antibody against β-galactosidase (Abcam) at 1:2000 dilution overnight at 4 °C. After washing with PBS, the slides were incubated with Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 anti-mouse IgG (Invitrogen) diluted 1:200 with PBS for 30 min at room temperature. Hoechst dye 33258 was used to label nuclei. The sections were washed and examined using a Nikon fluorescence microscope.

Immunohistochemistry. After blocking with 10% normal serum or BSA for 30 min, the sections were incubated with primary antibody against the antibody to HMGB2 (BD PharMingen) at 2 μg/mL and β-galactosidase (Abcam) at 1:2,000 dilution overnight at 4 °C. After washing the sections 3 times for 5 min in PBS, a second blocking was performed for 10 min. The sections were then incubated for 30 min with diluted biotinylated secondary antibody. The slides were washed 3 times in PBS and incubated for 30 min with Vectastain ABC-AP reagent or the peroxidase-based Elite ABC system (Vector Laboratories). The slides were washed, and the sections incubated for 4–20 min with alkaline phosphatase substrate solution or with 3,3 -diaminobenzidine (DAB) substrate. Slides were counterstained with diluted hematoxylin, rehydrated in 3 changes of PRO-PAR clearant, and mounted with Refrax mounting medium (Anatech).

Luciferase Reporter Assay. Cyclin D1 promoter vector (gift from O. Tetsu, University of California, San Francisco), TOPflash and FOPflash (gift from P.K. Vogt, The Scripps Research Institute) and Super8XTOPflash and Super8XFOPflash vectors (gift from R. Moon, University of Washington, Seattle) were used as reporter constructs for investigating the activity of Wnt/β-catenin signaling. Mutant β-catenin expression vector and pHMGB2 vector were gift from O. Tetsu and M. E. Bianchi (San Raffaele University, Milan), respectively.

Human SW1353 chondrosarcoma cells and 293T human embryonic kidney cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics. Murine chondrogenic ATDC5 cells were grown in DMEM/F-12 medium supplemented with 5% heat-inactivated FCS and antibiotics. The cells were seeded in 48-well plates and transfected with one or two expression vectors (HMGB2, β-catenin; 50 ng of each) and reporter constructs (cyclin D1, TOPflash or FOPflash; 50 ng of each) using FuGENE HD (Roche Molecular Biosystems). Luciferase activity was measured 24 h after transfection using a dual-luciferase reporter gene assay system (Promega), and the reporter activities were normalized to activity from a pRL family Renilla luciferase control vector (pRL-TK vector; Promega). In each experiment these assays were performed in triplicate. SuperTOPflash or SuperFOPflash reporter genes (150 ng of each) were transfected into murine WT or *Hmgb2*$^{-/-}$ chondrocytes (17) using Lipofectamine 2000 (Invitrogen). After 6 h, the chondrocytes were stimulated with or without recombinant murine Wnt3a (150 ng/mL; R&D Systems) for 8 h, then luciferase activity was measured as described above. These assays were performed in quadruplicate.

Small Interfering RNA (siRNA). TriFECTa Dicer-Substrate RNAi kit for murine HMGB2 was purchased from Integrated DNA Technologies. Murine articular chondrocytes were isolated as described in ref. 57, cultured in 6-well plates (2×10^5 per well), and transfected with 100 nM siRNA for HMGB2 (MMC.RNAI.N008252.5.3_2 nm and N008252.5.6_2 nm) or negative control (DS Scrambled-Neg, universal negative control duplex) using Oligofectamine (Invitrogen). Transfected cells were cultured for 48 h and collected for protein and RNA isolation. Expression of HMGB2 was examined by Western blot analysis (17), using anti-HMGB2 polyclonal antibody (BD PharMingen).

Quantitative PCR. The primers used in real-time PCR are as follows: Cyclin D1, 5′-GCCTACAGCCCTGTTACCTG (sense) and 5′-ATTTCATCCCTACCGCTGTG (antisense). Gli3, 5′-AGGAAGCGGACAAAGATGAA (sense) and 5′-GCTTTGAACGGTTTCTGCTC (antisense). Fzd2, 5′-CCGTCTCTGGATCCTCACAT (sense) and 5′-AGAAGCGCTCATTGCATACC (antisense). PRG4/SZP, 5′-CAAGAAGCCCACCTCTACCA (sense) and 5′-TTCAGGTGTTGCTGAAGTCG (antisense).

Chromatin Immunoprecipitation Assay. The ChIP assay was performed using the ChIP Assay Kit (Upstate) as described by the manufacturer with the following modifications. SW1353 cells with transfection of Lef-1 and HMGB2 or only Lef-1 were incubated in 100-mm dishes for 24 h when then the cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. The cells were lysed in 300 μL of SDS lysis buffer for 10 min on ice and sonicated three times for 10 sec. The sonicated samples were incubated with 4 μg of Lef-1 mouse monoclonal IgG or normal mouse IgG (Upstate), overnight at 4 °C. Immune complexes were washed following the manufacturer's instructions. Complexes were then eluted twice at room temperature for 15 min in elution buffer (1% SDS, 0.1M NaHCO3). Immunoprecipitated DNA was reverse cross-linked at 65 °C for 6 h in 200 mM NaCl and phenol/chloroform purified. An aliquot of the immunoprecipitated DNA (5 μL) and chromatin input control (1 μL) was used for PCR (35 cycles). All reactions were done under an annealing temperature of 58 °C. Two primers for amplifying Lef-1 binding sites in the human Gli3 enhancer (+23951 to +24251) were as follows: sense 5′-CAGCCCCAGGTAGGTATGTTG and antisense 5′-TTGATCCTTGCTGGCACAGA. All PCR products were evaluated on a 2% agarose gels for appropriate size (301 bp). As controls the human Gli3 promoter primers (−486 to −236: sense 5′-TCCACCCCGTGAGTAGTGC and antisense 5′-GCAGCTCCGGCATCAGTTT) were used to demonstrate the specificity of the immunoprecipitated chromatin.

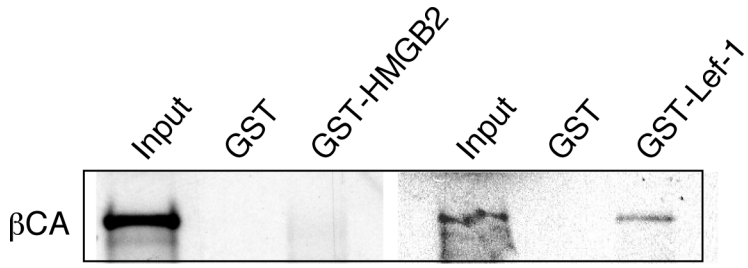

Fig. S1. GST-pull down assay for β-catenin (βCA), Lef-1 and HMGB2. In vitro-translated β-catenin interacts with GST-Lef-1, which was used as a positive control, but not with GST-HMGB2. Input was 20% of the sample used for pull-down assay.

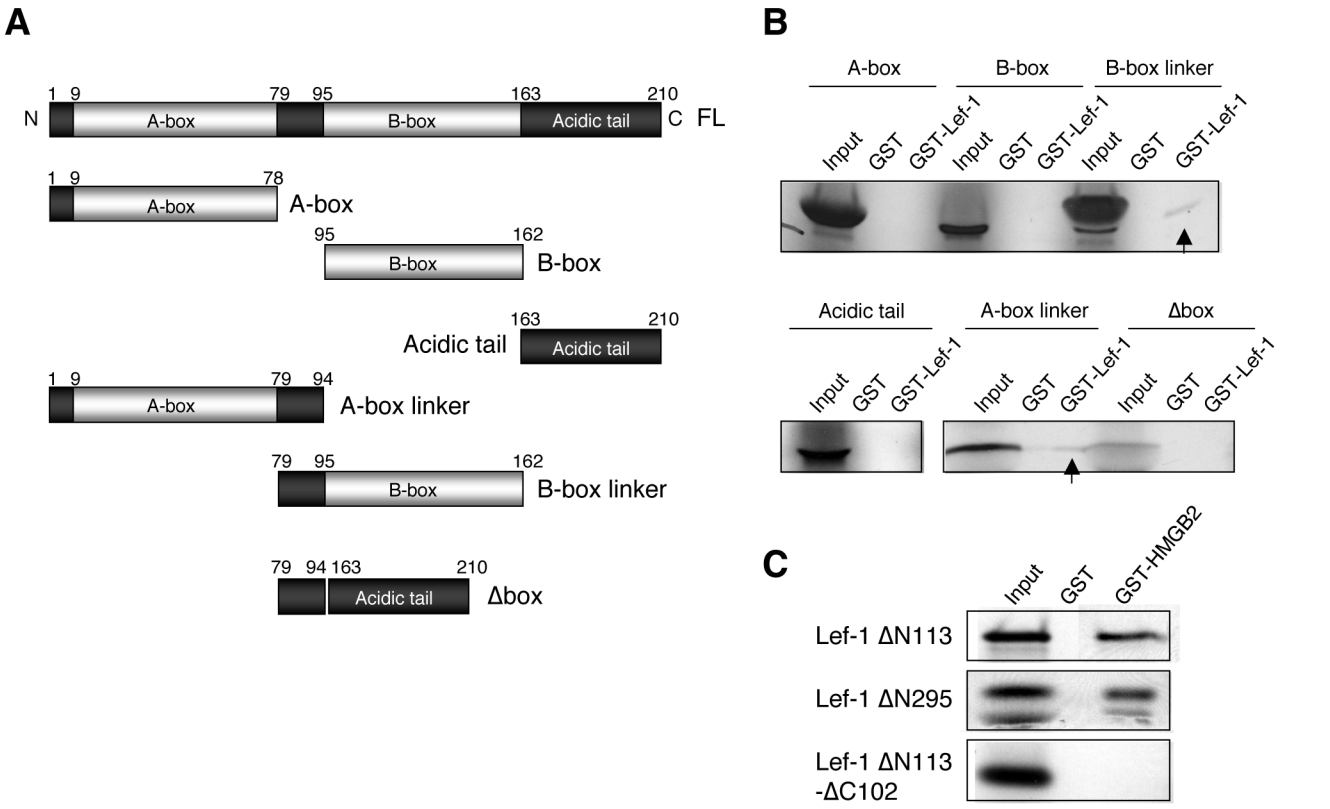

Fig. S2. Mapping of interaction domains of HMGB2 and Lef-1. Deletion mutants of in vitro-translated HMGB2 were incubated with either GST or GST-Lef-1 and pull down assays were performed to examine specific interactions. (*A*) The constructs were: full-length (FL) (amino acids: 1–210), A-box (1–78), B-box (95–162), C-terminal acidic tail (163–210), A-box-linker (1–94), B-box-linker (79–162), ΔBox (79–94;163–210). Only A-box linker and B-box linker can interact with Lef-1 (*B*, arrows). (*C*) Mapping of the interaction domain within Lef-1 by GST pull-down assay using GST-HMGB2 and in vitro-translated Lef-1 deletion mutant plasmids. The full-length Lef-1 cDNA encodes a protein of 397 residues and includes the β-catenin binding domain (bBD) in the N terminus. The Lef-1 Δ113 construct represents a smaller alternative Lef-1 transcript, which does not include the bBD in the N terminus. The Lef-1 Δ295 construct contains the HMG domain. Lef-1 ΔN113-ΔC102 includes only the context-dependent activation domain. GST-HMGB2 can bind Lef-1 ΔN113 and Lef-1 ΔN295, which contain HMG domain, but not ΔN113-ΔC102. Input was 20% of the sample used for pull-down assay.

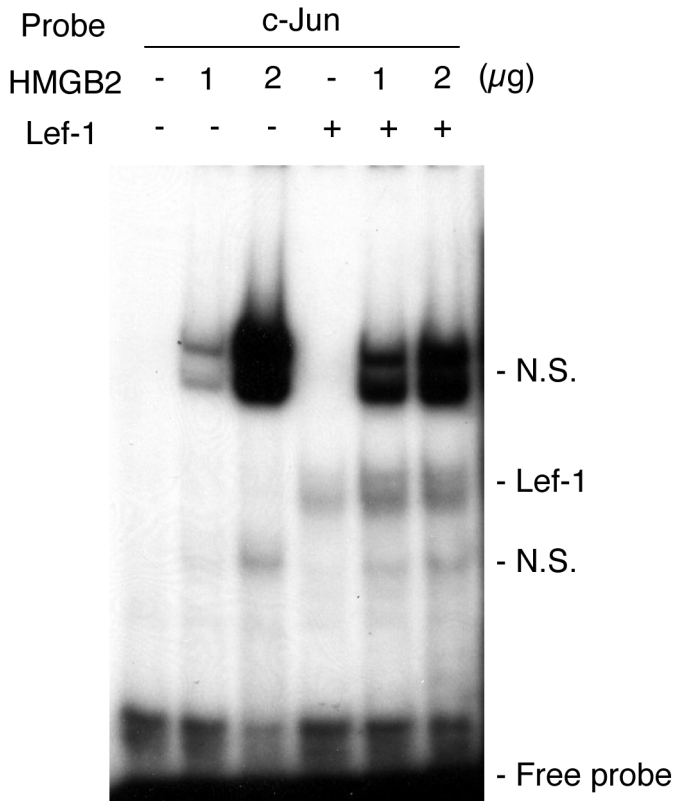

Fig. S3. DNA binding interactions of HMGB2 and Lef-1. Using nuclear extracts of Lef-1 transfected SW1353 chondrosarcoma cells, we detected binding of Lef-1 with c-Jun probes, and this binding was enhanced by the addition of purified HMGB2 (1–2 μg). N.S., nonspecific.

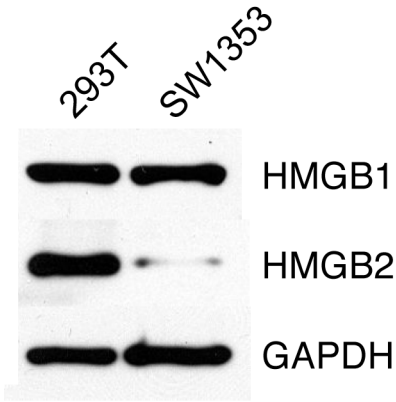

Fig. S4. HMGB1 and HMGB2 expression in 293T and SW1353 cells. Using cell lysates (10 μg) isolated from the semiconfluent cells, Western blot analysis was performed with chicken anti-HMGB1 antibody and rabbit anti-HMGB2 antibody. In 293T cells both HMGB1 and HMGB2 are abundant, whereas HMGB2 expression is much lower in SW1353 cells.

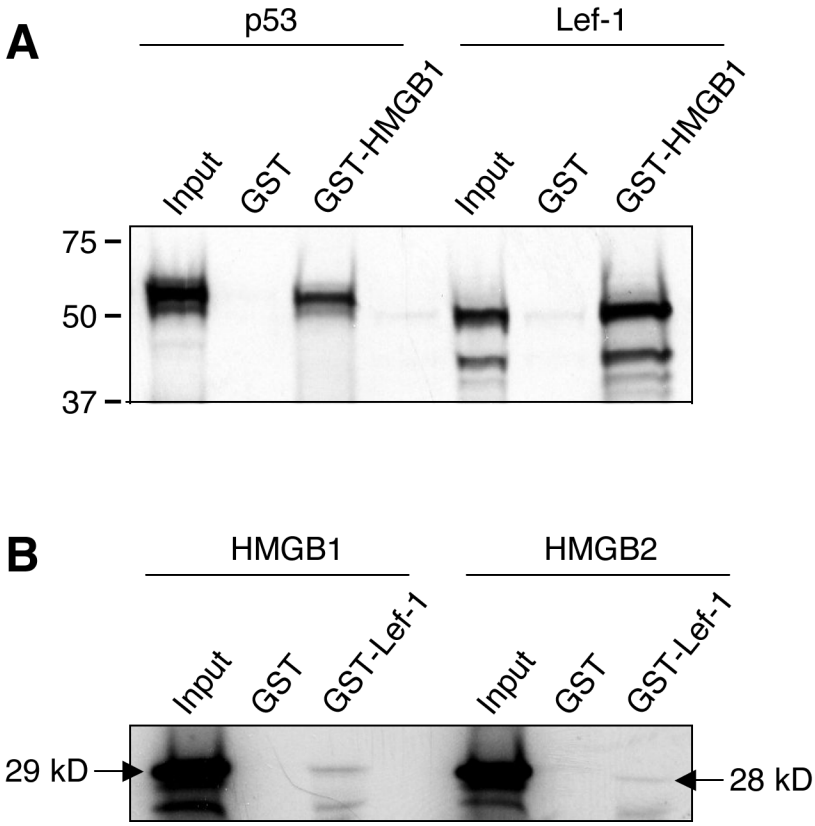

Fig. S5. Direct interaction of HMGB1 with Lef-1. (*A*) GST-pull down assay shows that GST-HMGB1 can interact with in vitro-translated Lef-1 and p53 that was used as a positive control. Input was 10% of the sample used for pull-down assay. Positions of molecular mass standards (in kilodaltons) are shown at the left. p53 expression vector was a gift from A. Nakagawara (Chiba Cancer Center Research Institute, Japan). (*B*) The reverse experiment shows that GST-Lef-1 binds to in vitro-translated HMGB1 and HMGB2. Input was 20% of the sample used for this assay.

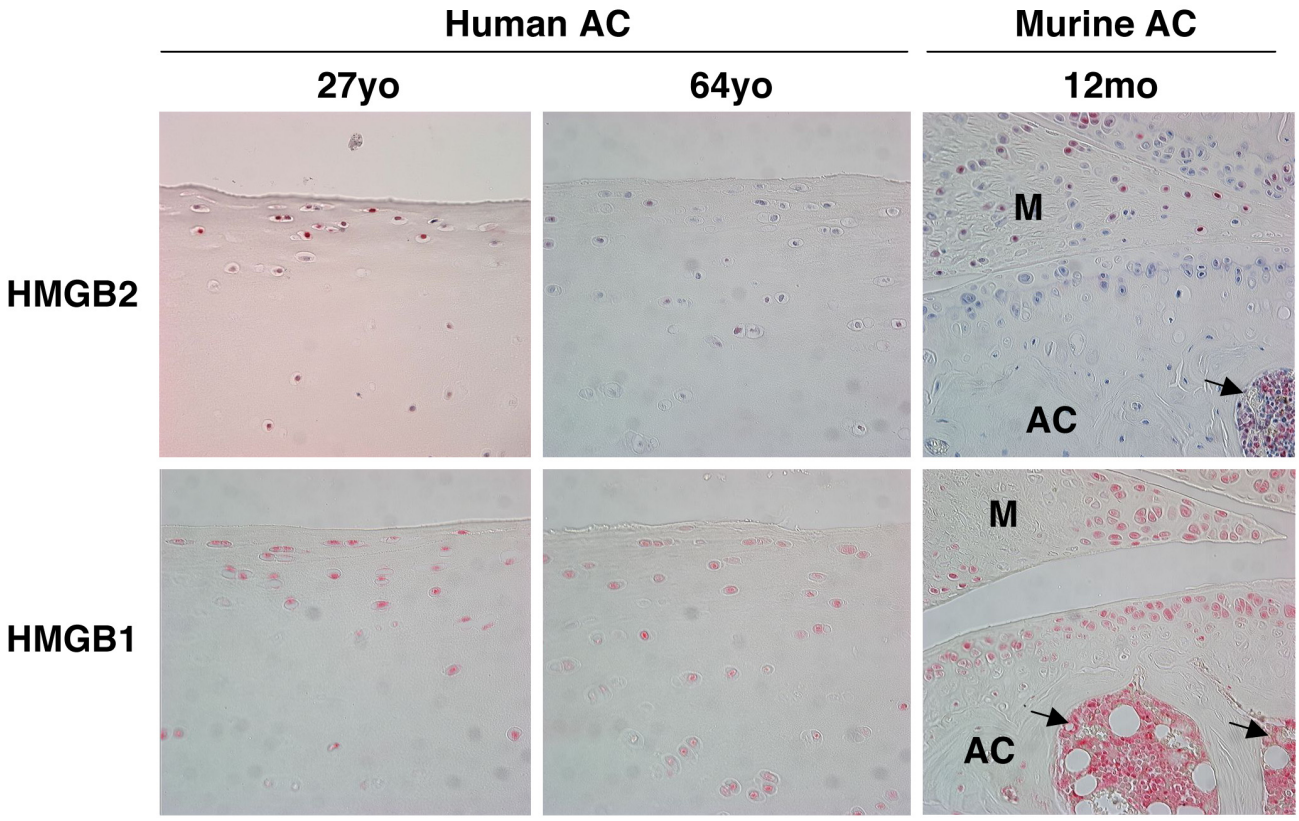

Fig. S6. Immunohistochemistry shows that HMGB2 is localized in the superficial zone of cartilage, whereas HMGB1 is expressed in the nuclei throughout normal articular cartilage from young donor aged 27 years. In old donor aged 64 years, HMGB2 is negative but HMGB1 is still positive. In 12-month-old mouse knee joints, HMGB2 is negative in articular cartilage, although bone marrow is positive (arrow). In contrast, HMGB1-positive cells are found in articular cartilage and bone marrow (arrows). AC, articular cartilage; M, meniscus. X400.