Supplemental Figures Legends

Supplemental.....Figure 1

A) Western Blot analysis of Runx2 (upper panel) and Actin in BCPAP, TPC1 and WRO thyroid tumor cells. B) qRT-PCR analysis of Runx2 mRNA levels in BCPAP cells after Runx2 and control siRNA transfection. Results were normalized to the GAPDH levels. The bars represent the averaged fold change of Runx2 in cells transfected with siRNAs against Runx2 as compared to the cells transfected with control siRNAs. C) Western blot analysis of Runx2 and Actin in BCPAP cells after Runx2 and control siRNAs transfection. D) Scratch wound healing assay. Light microscopy images of B-CPAP cells transfected with Runx2 or control siRNAs during a representative scratch wound healing assay at time 0, 12h and 24h after scratch application. 100X Magnification. Four scratches for each well were analyzed in this experiment obtaining the same results. E) qRT-PCR analysis of the MMP2, MMP13, MMP14, TIMP3 and OPN in BCPAP cells transfected with siRNAs against Runx2 (white bars) or control siRNAs (black bars). Results were normalized to the GAPDH levels. The bars represent the averaged fold change of indicated mRNAs in cells transfected with siRNA against Runx2 as compared to the cells transfected with control siRNAs. ***$p \leq 0.001$; **$p \leq 0.01$.

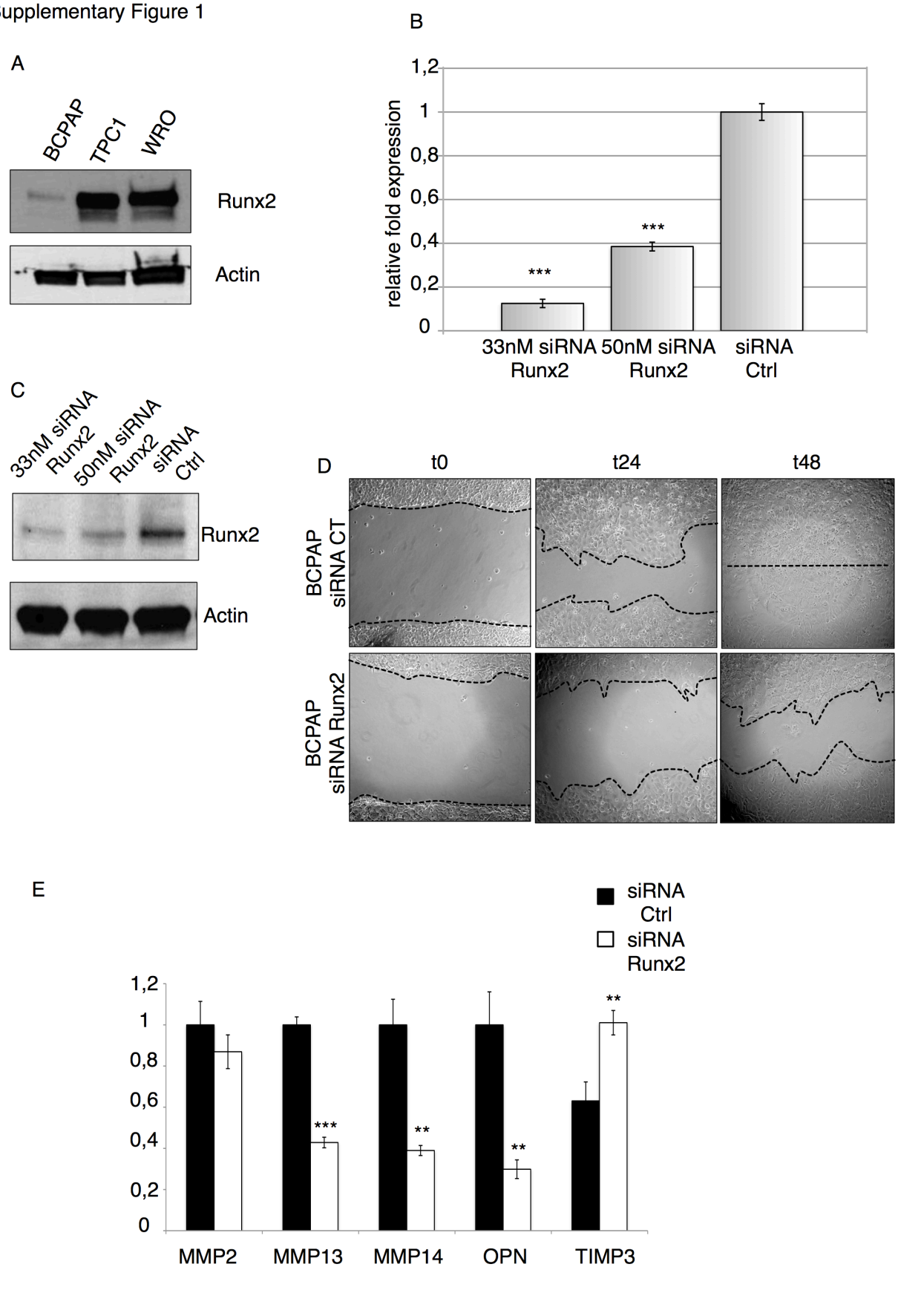
Supplementary Figure 2

A) qRT-PCR analysis of Runx2 mRNA levels in TPC1 cells after Runx2 and control siRNA transfection. Results were normalized to the GAPDH levels. The bars represent the averaged fold change of Runx2 in cells transfected with siRNAs against Runx2 as compared to the cells transfected with control siRNAs. B) Western blot analysis of Runx2 and Actin in TPC1 cells after Runx2 and control siRNAs transfection. C) Proliferation curves of TPC1 cells transfected with siRNAs against Runx2 or control siRNAs. Curves represent the averaged number of cells per well for each line at each time point ± SEM. D)Western blot analysis of p21 levels in Rx12, Rx21 and control cells. E) Quantification of the Western blot signals. The histograms represent the averaged percentage of p21 relative to control cells. F) Immunofluorescent staining with anti-p21 antibody (red) in Rx12, Rx21 and control cells. DAPI in blue (right panels). Magnification 400X. G) Immunohistochemistry staining of Runx2 (brown) in Runx2 overexpressing (upper) and control (lower) cells. F) Immunohistochemistry staining of Runx2 (brown) in normal human thyroid tissues.

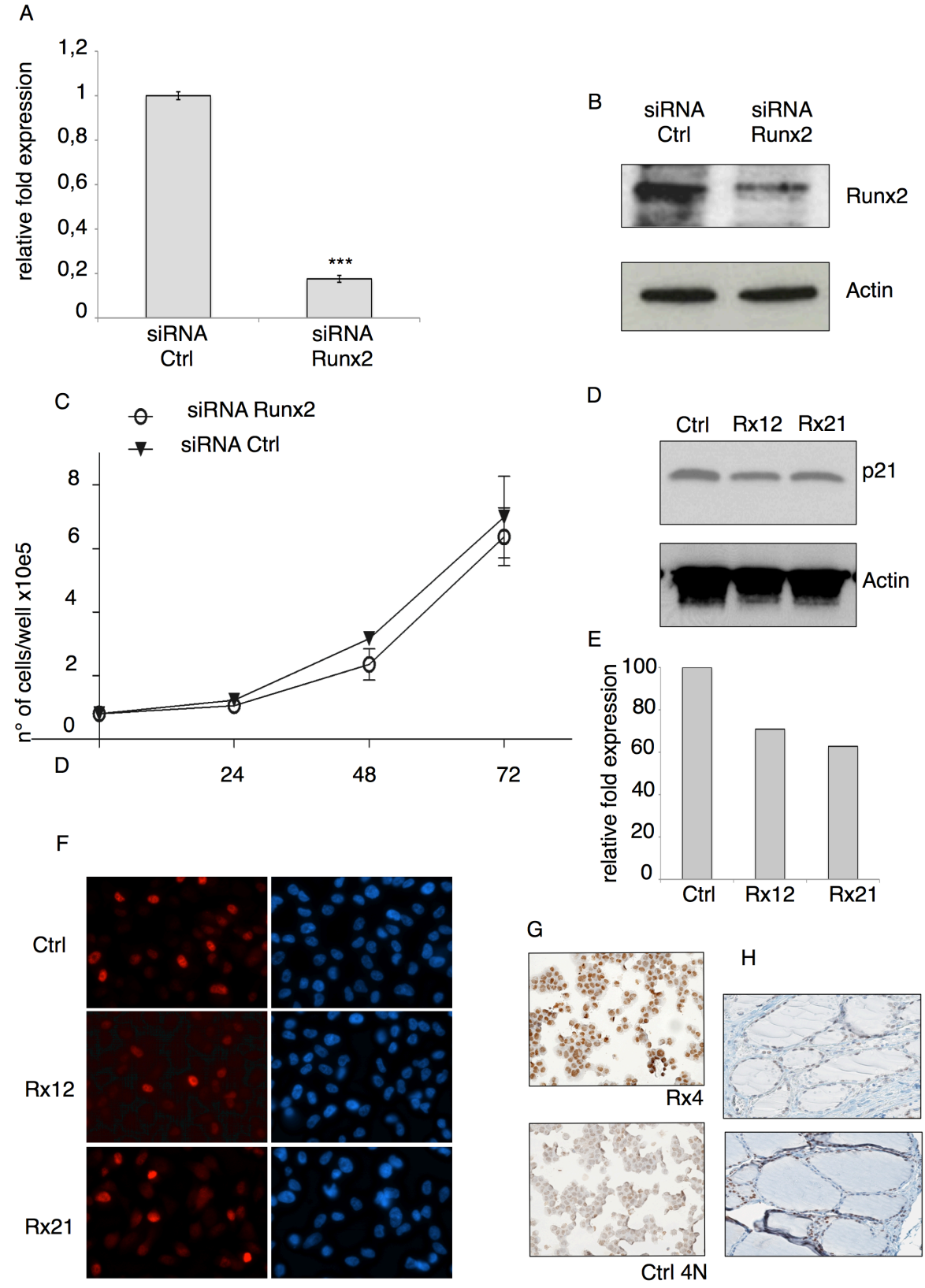
Supplementary Figure 3

qRT-PCR analysis of MMP2, MMP13, MMP14, OPN and TIMP3 mRNA levels in normal tissue (white bars) and primary tumors (black bars) from 8 PTC patients. No reliable amplification signal was obtained for MMP13 in PTC6 and 7 both in the primary tumor and normal tissue. The bars represent the averaged fold change of the indicated mRNA in primary tumor as compared to normal tissue. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$.

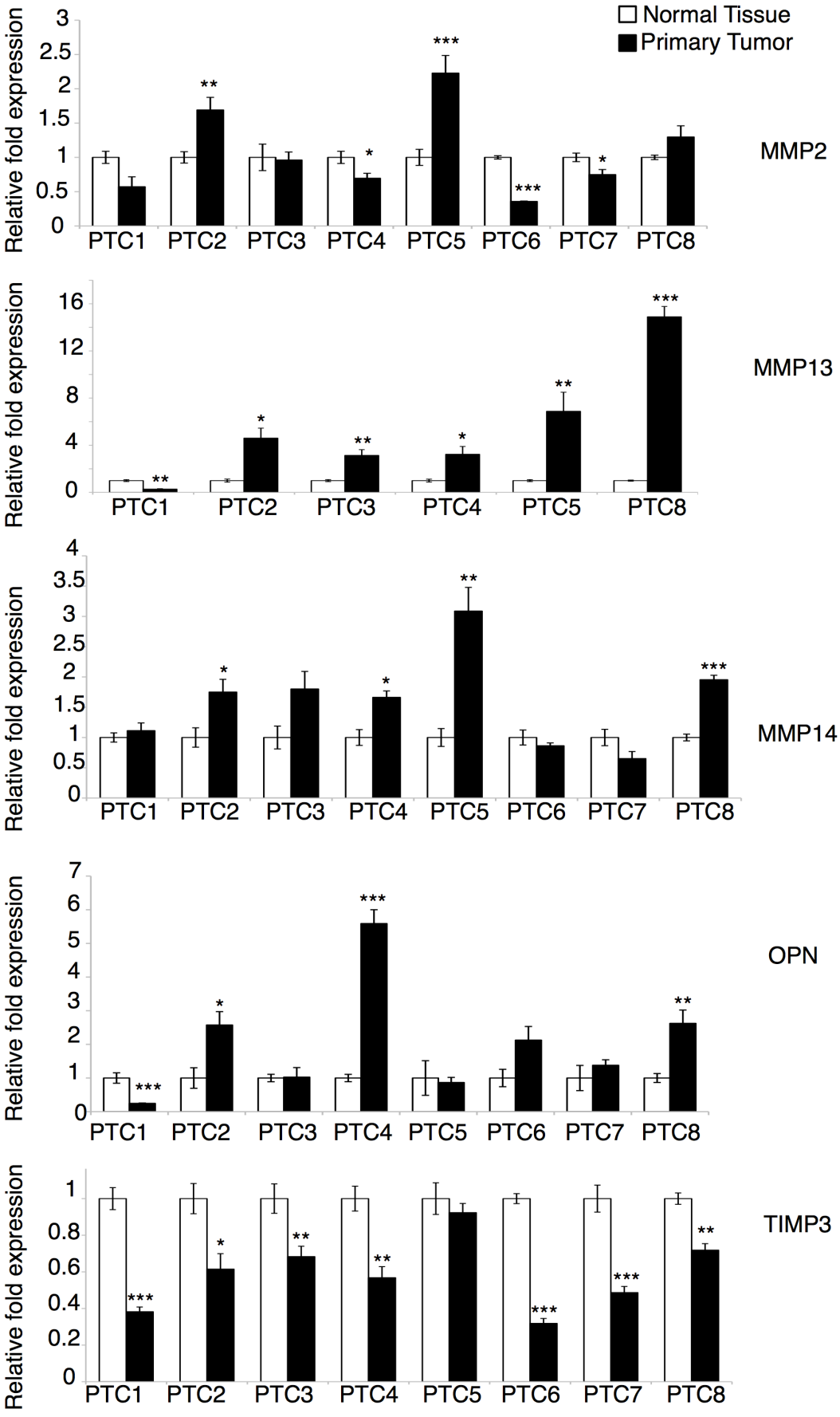
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Methods

Runx2 stable clones derivation

pCDNA 3.1 vector was purchased from Invitrogen. Runx2 isoform I cDNA was amplified from total mRNA extracted from B-CPAP cells after retrotranscription with Oligo dT using the Prime Script cloning kit (Takara). For Runx2 isoform I amplification the following primers were used: Runx2-EcoRV-F gcagatatcatgcgtattcccgtag; Runx2-XhoI-R gtcctcgagtcaatatggtcgcca. For stable clone derivation, B-CPAP cells were transfected with Runx2 isoform I-pCDNA 3.1 or with pCDNA 3.1 plasmids using Lipofectamine 2000 (Invitrogen, Italy). Twenty-four hours after transfection, cells were seeded at very low density and Geneticin (Invitrogen, Italy) was added to the regular growth medium at a concentration of 800 μg/ml. Three weeks after selection, single clones were picked and expanded. Forty-three Runx2 overexpressing clones and 10 control clones were obtained and characterized for Runx2 overexpression by Western blot and qRT-PCR.

Quantitative Real Time PCR

Total RNA purification from cells was performed with RNAeasy Mini kit (Qiagen, Italy). 1μg of total RNA was retrostrancribed using the iScript cDNA kit (Biorad, Italy). qRT-PCR was conducted using Sso Fast EvaGreen Super Mix (BioRad, Italy) in the CFX96 Real Time PCR Detection System (BioRad, Italy). Relative expression of target genes was calculated using the ΔΔCT method and normalized to GAPDH mRNA content.

For semiquantitative RT-PCR, samples were processed as described above. End-point PCR products were analyzed by electrophoresis on 2% agarose gel and analyzed using the Gel-Doc System (BioRad, Italy). Sequences of primers are listed in Supplementary Table 2.

RNA extraction from formalin-fixed paraffine-embedded tissues

Total RNA was collected from 5 slices, 5μm thick, of formalin-fixed and paraffin-embedded PTCs, using the High Pure RNA paraffin kit (Roche, Italy). All samples (normal, primary tumor and metastasis) were manually dissected under microscopic guidance by the Pathologists MR and SP. In order to minimize the biases that a different tissue processing could introduce on RNA quality, we performed gene expression analysis comparing normal tissue, primary tumor and metastasis from the same patient. Primary tumor and normal tissues were collected from the same slide. Quantity and purity of the total RNAs were checked using the NanoDrop 2000

spectrophotometer. Differences in the reference gene levels among the normal and tumor samples from the same patient were minimal (ΔCT<0.5 cycle).
Primers for qRT-PCR were designed in order to obtain amplicons less than 100bp in length.

Immunofluorescence

Cells were seeded in 4 well Lab-Tek Chamber slides (Nunc) in regular growth medium. Twenty-four hours after seeding, cells were fixed in 4% PFA in PBS1X for 15 minutes at room temperature, permeabilized with 0,1% Triton in PBS1X for 2 minutes, blocked with 20%FBS and 2%BSA in PBS1x for 1 hour, then incubated with a goat anti-Runx2 antibody (R&D System, Italy) or a rabbit anti-p21 antibody (Santa Cruz, USA) in a humidified chamber for one hour. Antibody binding was revealed with a secondary anti-goat Alexa 488 conjugated antibody (Invitrogen, Italy) or anti-rabbit Alexa 594 (Invitrogen, Italy). Cell nuclei were stained with DAPI (Invitrogen). Slides were mounted using the SlowFade mounting medium (Invitrogen) and observed using a Axiophot fluorescent microscope (Zeiss).

DNAse sensitivity assay

Chromatin accessibility of the P1 and P2 promoters was assessed by using the EpiQ Chromatin Analysis kit (Biorad) according to manufacturer protocol. Briefly, the Id1A, Id1B and ct3 cells were seeded in a 48-well plate and the following day were treated with EpiQ nuclease or chromatin buffer. Genomic DNA was extracted and integrity of P1 and P2 promoter regions was measured by quantitative PCR using the EpiQ chromatin SYBR Supermix (Biorad) and the CFX96 Real Time PCR Detection System (Biorad). Sequence of primers used are listed in Supplementary Table 2. For each experimental point both a biological triplicate and a technical triplicate were performed. Accessibility of the two promoters was estimated by comparing the EpiQ nuclease-treated samples and the undigested control samples using the EpiQ chromatin kit data analysis tool (Biorad).

Statistical Analysis

All statistical analysis were performed using an unpaired t-test using GraphPad Prism Software.

Supplemental Table 1 . Primer sequences

Runx2 Isoform II dir	GCACAGTGACACCATGTCAGC
Runx2 Isoform I dir	GATGCGTATTCCCGTAGATCC
Runx2 Isoform I rev e Runx Isoform II rev	CTGTTGCGCAGCCACCAC
Runx2 dir	GTGCCTAGGCGCATTTCA
Runx2 rev	GCTCTTCTTACTGAGAGTGGAAGG
Gapdh dir	ATTGGGCGCCTGGTCAC
Gapdh rev	AAGATGTAAACCATGTAGTTGAGGTCA
MMP13 dir	CTTCAAAGTTTGGTCCGATGT
MMP13 rev	GTAGAAGCTGCCATGCTCCT
MMP2 dir	ATAACCTGGATGCCGTCGT
MMP2 rev	TCACGCTCTTCAGACTTTGG
MMP14 dir	TACTTCCCAGGCCCCAAC
MMP14 rev	GCCACCAGGAAGATGTCATT
TIMP3 dir	CCTTCTGCAACTCCCGACATC
TIMP3 rev	TGATGGTGTAGACCAGCGTG
OPN dir	CGCAGACCTGACATCCAGT
OPN rev	GGCTGTCCCAATCAGAAGG
P21 dir	GCCTGGACTGTTTTCTCTCG
P21 rev	ATTCAGATGTGGGAGGAG
EpiQ_P1 for	GGAGTTTGGGCTCCTTCAGCATTTGT
EpiQ_P1 rev	AGGCGAATGAAGCATTCACACAATCCA
EpiQ_P2 for	GAGGTGGCTGTGAGAGGGCGAGAAG
EpiQ_P2 rev	GTGAAGCGGTGTGGGTTCAGCAGTC