Supporting Information

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SI Materials and Methods

Cell Culture and Cell Synchronization. Human osteosarcoma (SaOS-2) cells (American Type Culture Collection) were grown in HyClone McCoy's 5A medium containing 1.5 mL glutamine and 2.2 g/L sodium bicarbonate (Thermo Scientific HyClone Laboratories) containing 15% FBS premium (Atlanta Biological), 2 mM pencillin streptomycin, and 20 mM L-glutamine was added (Invitrogen). To synchronize the cells in mitosis, cells were treated with 100 ng/mL nocodazole for 18 h. After 18 h, cells were washed with 1× PBS twice and fresh media was added to release cells from the nocodazole block. Progression of cells through the cell cycle was confirmed by fluorescence-activated cell sorting (FACS) for DNA content and Western blot in the absence and presence of the protein synthesis inhibitor cycloheximide as described (1).

Primary and Secondary Antibodies. The following antibodies were used in this study: Runx2 (M-70) rabbit polyclonal antibody (Santa Cruz Biotechnology) and Runx2 (8G5) mouse monoclonal antibody (MBL International). Dilutions and concentration used for M-70 in IF (1:500), IP (4 μg), and ChIP (6 μg) and for 8G5 in WB (1:2,000). UBF (F-9) mouse monoclonal antibody (Santa Cruz Biotechnology). Dilutions used for IF (1:400), WB (1:1,000). UBF rabbit polyclonal antibody (Santa Cruz Biotechnology). Concentrations used for IP (4 μg) and ChIP (6 μg). TLE1 rabbit polyclonal antibody (Abcam). Dilutions and concentration used for IF (1:500), WB (1:600), and ChIP (6 μg). HDAC1 (H-51) rabbit polyclonal antibody (Santa Cruz Biotechnology). Dilutions used for IF (1:1,000). HDAC1 mouse monoclonal antibody (Upstate Biotechnology). Dilution used for WB (1:4,000). HDAC1 rabbit polyclonal antibody (Abcam). Concentration used for ChIP (6 μg). CdK2 M2, CyclinE, and CyclinB rabbit polyclonal antibodies (Santa Cruz Biotechnology) dilutions used for WB (1:1,000). LaminB1 mouse monoclonal (Zymed Laboratories). Dilutions for WB (1:4,000). Nucleophosmin B23 mouse antibody (Zymed Laboratories). Dilution used for WB (1:16,000). Mouse and Rabbit normal IgG antibodies (Santa Cruz Biotechnology). Concentrations for IP (4 μg) and ChIP (6 μg). Rabbit Anti-Flag Polyclonal Antibody unconjugated or Anti-Flag M2 mouse monoclonal antibody (Sigma). Dilution and concentration used for WB (1:2,000), IP (4 μg) and ChIP (6 μg). Histone modification antibodies for H3 acetylation, H4 acetylation and H3k9 acetylation (Upstate Biotechnology). Secondary horseradish peroxidase (HRP)-conjugated Goat anti-mouse and rabbit antibodies (Santa Cruz Biotechnology). Dilution for WB (1:2,000). Secondary peroxidase-conjugated Goat Anti-Mouse IgG for WB (1:2,000) (Jackson ImmunoResearch Laboratories). Monoclonal Anti-flag M2-Peroxidase (HRP) (Sigma). Dilution for WB (1:2,000). The secondary antibodies for IF used were either goat anti-mouse Alexa 594 or goat anti-rabbit Alexa 488 (1:800) Molecular Probes).

In Situ Immunofluorescence Microscopy. SaOS-2 cells, grown on gelatin-coated coverslips, were processed for in situ immunofluorescence as described (1, 2). In brief, cells were fixed in 3.7% formaldehyde 10 min on ice. Fixed cells were permeabilized in 0.1% Triton X-100 in PBS, followed by 1 h of incubation with primary antibody at 37 °C. Cells were washed with PBSA and incubated with secondary antibody at 37 °C. DNA was visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. Images were analyzed and captured by using an Epifluorescence Zeiss Axioplan 2 (Carl Zeiss MicroImaging) microscope equipped with a charged coupled device and MetaMorph Imaging Software (Molecular Devices).

Coimmunoprecipitation and Western Blot Analysis. Confluent SaOS-2 cells were washed twice with ice-cold PBS and harvested in cold sonication buffer [50 mM NaCl, 50 mM Tris (pH 8.0), 1% NP-40, 25 mM MG132, and 1× protease inhibitor mixture (Roche)]. Cells were sonicated by using Fisher Scientific Sonic Dismembrator 550 fitted with a 1.6-mm tip (Fisher Scientific) and then centrifuged (16,000 × *g* for 10 min). Lysates were incubated overnight at 4 °C with 4 μg of antibodies. Lysates were then incubated with protein A/G beads (Santa Cruz Biotechnology) for 2 h, followed by 4 washes with ice cold wash buffer [50 mM NaCl, 20 mM Tris (pH 8.3), 0.5% Na-10 deoxycholate, 0.5% Nonidet P-40, 2 mM EDTA, 25 μM MG132, and 1× protease inhibitor mixture]. The total cell lysate as well as immunoprecipitated protein complexes were resolved by 8% SDS/PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Blots were incubated with different primary antibodies followed by incubation with HRP conjugated secondary antibodies. Proteins bands were visualized by a chemiluminescence detection kit (Perkin-Elmer Life Sciences).

Chromatin Immunoprecipitation (ChIP) and ChIP ReChIP. ChIP assays were performed essentially as described (1). Briefly, cells were cross-linked in serum-free DMEM with 1% formaldehyde for 10 min, and the reaction was quenched by glycine (250 mM) for 10 min. Cells were washed with PBS and were harvested in lysis buffer [150 mM NaCl, 50 mM Tris·HCl, (pH 8.0), 1% Nonidet P-40, 25 μM MG-132, and 1× Complete protease inhibitor mixture (Roche)]. After 10 min of incubation on ice, cells were sonicated to generate ≈500–600 base pairs of DNA fragments. Cell debris was removed by centrifugation at 16,000 × *g* for 20 min. Two percent of the supernatant was kept as input. The rest of the supernatant containing protein–DNA complexes were incubated overnight with 6 μg of specific and normal IgG antibodies, followed by 2 h of incubation with protein A/G-conjugated agarose beads. Protein A/G bead complexes were washed with the following buffers: low salt [20 mM Tris·Cl, (pH 8.1), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, and 1× Complete protease inhibitor mixture (Roche)], high salt (20 mM Tris·Cl, (pH 8.1), 500 mM NaCl, 1% Triton X-100, 2 mM EDTA], LiCl [10 mM Tris·Cl, (pH 8.1), 250 mM LiCl, 1% deoxycholate, 1% Nonidet P-40, 1 mM EDTA], and twice in TE [10 mM Tris·Cl, (pH 8.1), 1 mM EDTA] protein–DNA complexes were eluted in 1% SDS/100 mM $NaHCO_3$ (elution buffer) and cross-linking was reversed by incubation at 65 °C for overnight in elution buffer and 5 M NaCl (200 mM). DNA was extracted, purified, and precipitated by phenol-chloroform and ethanol precipitation and then resuspended in TE for quantitative PCR analysis. ChIP enrichment was determined as a quantitative measure reflecting the percentage of input. Human rDNA primer sets A, B, and C as well as nonspecific Phox (GP91) were used as mentioned (1). ChIP ReChIP experiments were carried out essentially as described (3). Briefly, immunoprecipitates from the first ChIP were eluted in 10 mM DTT buffer for 30 min at 37 °C, diluted in ChIP dilution buffer and subjected to a second immunoprecipitation (i.e., ReChIP).

RNA Isolation and Quantitative PCR Analysis. Total RNA was isolated from SaOS-2 cells by using TRIzol reagent (Invitrogen) and purified using the DNase-free RNA kit (Zymo Research). CDNA (cDNA) was generated from purified RNA by using a reverse transcription reaction with random hexamer primers (Invitrogen)

and subjected to quantitative PCR by using SYBR-green chemistry (Applied Biosystems) and gene-specific primers.

RNA Interference. Small interfering RNA (siRNA) transfection for Runx2, TLE1, and HDAC1 was performed according to standard techniques by using Oligofactamine reagent (Invitrogen). SaOS-2 cells grown at 60% confluence were transfected with 40 nmol of either Runx2 smart pool RNAi or by using three independent predesigned TLE1 RNAi oligos (Dharmacon) for 48 h.

Runx2 Lentiviral Constructs. Wild-type and mutant Runx2 lentiviral particles were generated by using Lentiviral Gateway System (Invitrogen). Wild-type and mutant Runx2 cDNAs were cloned into pENTR4-FLAG vector and then recombined with lentiviral destination pLenti-CMV-Blast-DEST vector by using Gateway LR clonase enzyme mix according to manufacturer specifications. Lentiviral particles were generated in 293T cells by transient transfection of Runx2 constructs along with packaging plasmids. Viral particles were collected after 48 h of transfection and used for infecting SaOS-2 cells.

Proliferation Assays. Equal numbers of cells were transfected with either nonspecific or specific RNAi Oligos against Runx2 and TLE1. Cells were collected up to 96 h after every 12 h by trypsinizing in the SaOS-2 media. Equal volume (20 μL) of suspension was loaded on Nexcelom Cellumeter glass slides (Nexcelom Bioscience LLC). Cells were counted by using Cellometer Auto T4 Cellcounter (Nexcelom Bioscience LLC).

Metabolic Labeling. Metabolic labeling was performed as described (4). Briefly, SaOS-2 cells, transfected with RNAi against Runx2, TLE1 alone or both for 48 h were incubated in methionine-free RMPI media with 10% dialyzed serum for 1 h followed by incubation with EasyTag Express [^{35}S] protein labeling mix (200 μCu/mL) for 45 min (Perkin-Elmer). Cells were harvested in equal volume of direct lysis buffer [2 M urea, 2% SDS, 10 mM DTT, 10% glycerol, 10 mM Tris·HCl (pH 6.8), 0.2 mg/mL bromophenol blue, 1× complete pellet protease inhibitor (Roche), and 25 μM Mg132]. Proteins were separated by SDS/PAGE (8%). Gel was dried and exposed to scientific imaging film MR (Eastman Kodak) overnight at −70 °C.

1. Young DW, et al. (2007) Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2. *Nature* 445:442–446.
2. Young DW, et al. (2007) Mitotic retention of gene expression patterns by the cell fate-determining transcription factor Runx2. *Proc Natl Acad Sci USA* 104: 3189–3194.
3. Hovhannisyan H, et al. (2003) Maintenance of open chromatin and selective genomic occupancy at the cell cycle-regulated histone H4 promoter during differentiation of HL-60 promyelocytic leukemia cells. *Mol Cell Biol* 23:1460–1469.
4. Grandori C, et al. (2005) c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* 7:311–318.

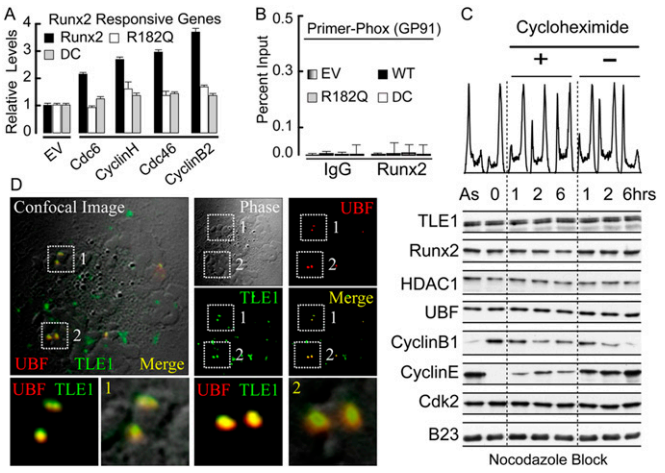

Fig. S1. (*A*) Runx2 expression positively regulates Pol II-transcribed genes in SaOS-2 cells as assessed by real-time qPCR. The Runx2 mutant R182Q and ΔC do not affect transcript levels of Pol II-mediated transcribed genes relative to internal control mCox gene. (*B*) Phox (GP91) promoter that does not have a Runx2 binding site was also amplified as a control for specificity. As expected Runx2, R182Q, and ΔC in ChIP analysis did not show any occupancy of the promoter. (*C*) SaOS-2 cells were synchronized in mitosis by using nocodazole in the presence and absence of cycloheximide to inhibit the protein translation. FACS analysis and DNA content was used to monitor the cell cycle progression after release from Nocodazole block. The protein levels of UBF, HDAC1, TLE1, and Runx2 remained unchanged in the absence or presence of cycloheximide during different time points as quantified by Western blot analysis. Stages of cell cycle were further analyzed by the temporal expression of various cyclins; CdK2 and B23 were used as loading controls. As expected CyclinB1 levels were diminished following mitosis, with a concomitant increase in CyclinE levels. (*D*) Confocal microscopy analysis of SaOS-2 cells metaphase spread confirms TLE1 colocalization with UBF at mitotic NORs.

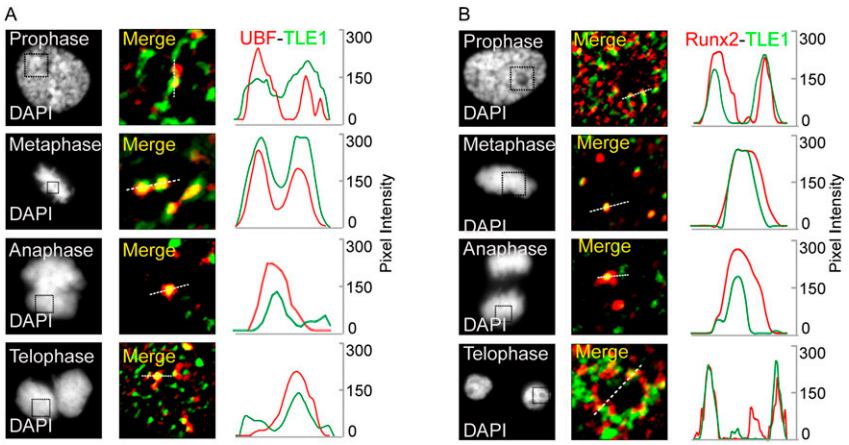

Fig. S2. (*A*) Immunofluorescence in SaOS-2 cells demonstrates colocalization of UBF and TLE1 during prophase, metaphase, anaphase, and telophase. DAPI images demonstrate the different mitotic stages as analyzed by microscopy. Black dotted square represents the region where TLE1 and UBF colocalize. Line scans were performed on foci where UBF and TLE1 show colocalization. (*B*) Immunofluorescence images show the colocalization of Runx2 and TLE1 foci during different stages of mitosis.

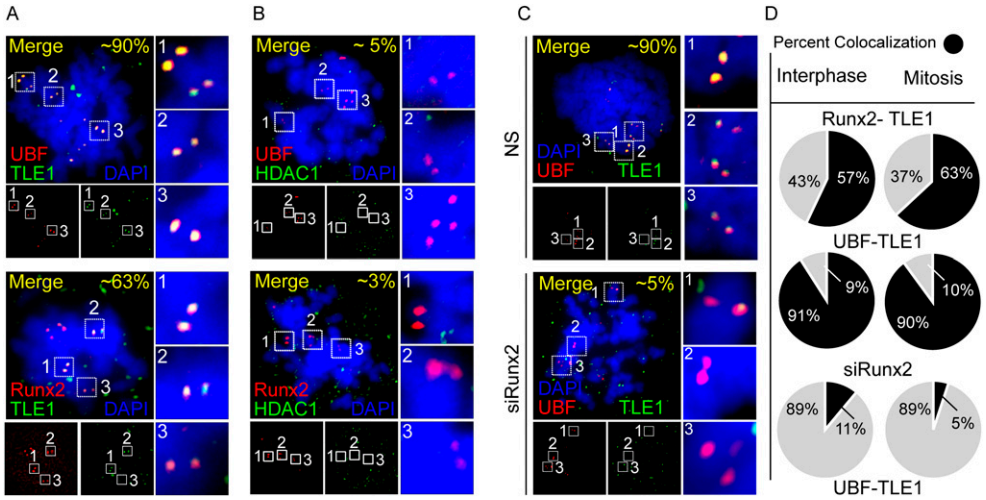

Fig. S3. (*A*) Metaphase spreads of SaOS cells demonstrate TLE1 colocalization with UBF (*Upper*) and Runx2 (*Lower*) at NORs. White dotted squares represent the NOR regions where TLE1 show colocalization with UBF and Runx2. (*B*) Immunoflorescence analysis of SaOS cells metaphase spreads shows HDAC1 foci do not colocalize with UBF (*Upper*) or Runx2 (*Lower*) at mitotic NORs. White dotted boxes represent the mitotic NOR regions. (*C*) Microscopy analysis of metaphase spreads of SaOS-2 cells show a decrease in colocalization of TLE1 and UBF at NORs in the absence of Runx2 (Fig. 4*B*, NS and siRunx2) when compared with nonspecific (NS) RNAi. White square boxes mark the NORs. (*D*) Pie graphs demonstrate the percent colocalization of Runx2, TLE1 and UBF during mitosis and interphase. Graphs also show the difference in UBF and TLE1 colocalization in the presence and absence of Runx2 in SaOS-2 cells as analyzed by microscopy.

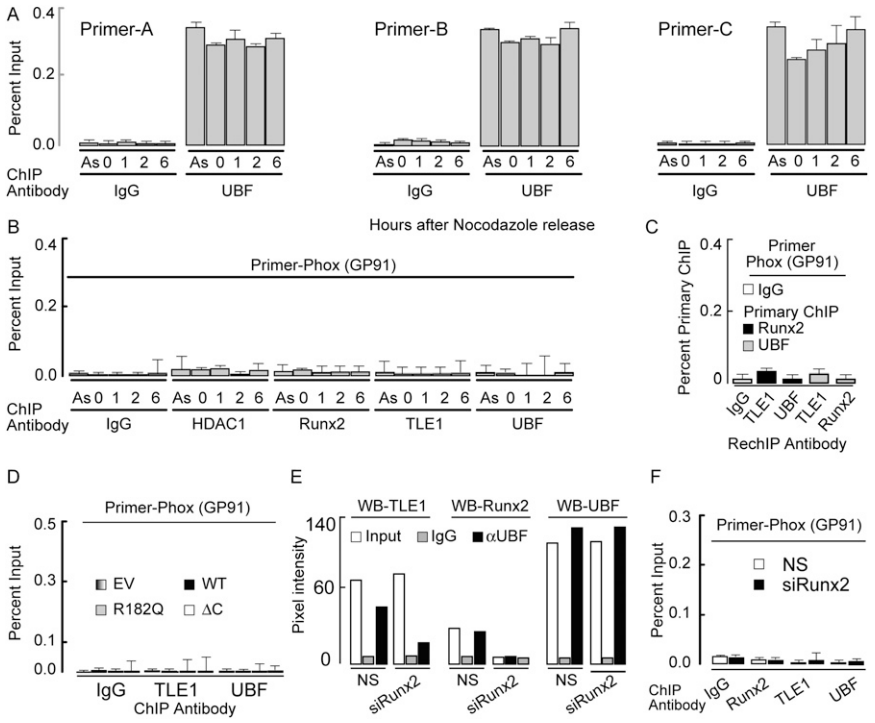

Fig. S4. (*A*) SaOS-2 cells were blocked in mitosis by using Nocodazole and then released to assess the changes in UBF binding at rDNA during different time points of the cell cycle by ChIP assays. (*B*) Phox (GP91) primer shows no occupancy of TLE1, Runx2, HDAC1, and UBF at Phox (GP91) gene promoter during all time points of the cell cycle. (*C*) Chromatin Immunoprecipitation-ReChIP assay demonstrates that neither Runx2 nor UBF occupy Phox (GP91) promoter. (*D*) Expression of WT-Runx or its mutants R182Q and ΔC does not enhance the binding of TLE1 or UBF at Phox (GP91) region as assessed by ChIP. (*E*) Quantitation of the Western blots (Fig. 4*A*) by using ImageJ software indicates a decrease in UBF interaction with TLE1 in the absence of Runx2 when compared to non-specific (NS) siRNA oligonucleotides. (*F*) Amplification of the Phox (GP91) promoter shows ChIP specificity as none of the factors analyzed bind to the Phox (GP91) region in the presence of Runx2 (NS) or by knocking down Runx 2 (siRNA).

Table S1. Primers used in study

Name	Sequence 5′ to 3′	Description
Primer A	Forward: AGGTGTCCGTGTCCGTGT Reverse: GGACAGCGTGTCAGCAATAA	Human rDNA ID: U13369
Primer B	Forward: TGTCAGGCGTTCTCGTCTC Reverse: GAGAGCACGACGTCACCAC	Human rDNA ID: U13369
Primer C	Forward: GGATGCGTGCATTTATCAGA Reverse: GTTGATAGGGCAGACGTTCG	Human rDNA ID: U13369
Phox (GP91)	Forward: CCAATGATTATTAGCCAATTTCTG Reverse: CATGGTGGCAGAGGTTGAATGT	Human Cytochrome *b* (558) Beta Subunit ID: M66390
prerRNA	Forward: CCGCGCTCTACCTTACCTAC Reverse: GAGCGACCAAAGGAACCATA	ID: U13369
28S-rRNA	Forward: GAACTTTGAAGGCCGAAGTG Reverse: ATCTGAACCCGACTCCCTTT	ID: U13369
Cdc6	Forward: TGCTCTTGATCAGGCAGTTG Reverse: CCAAGAGCCCTGAAAGTGAC	NM_001254
Cdc46	Forward: ACTTACTCGCCGAGGAGACA Reverse: CTGCCTTTCCCAGACGTGTA	NM_006739
CyclinH	Forward: CCTCCAGGGCTGGAATTACT Reverse: CTTCAGATCTGGGTGGTTCA	NM_001239
CyclinB2	Forward: AACCAGAGCAGCACAAGTAGC Reverse: ACCCTTTGGAGCCAACTTTT	NM_004701
mCox	Forward: ACGAAATCAACAACCCCGTA Reverse: GGCAGAACGACTCGGTTATC	Mitochondrial cytochrome C oxidase subunit II gene. ID: AF378830.