METHODS

Fluorescence-activated cell sorting (FACS). FACS sorting of quiescent and cycling cells was performed as previously described[1].

Reconstituted transcription *in vitro*. *In vitro* transcription was performed as described in[2] with the following modifications: the DNA template was incubated in 20 µL with transcriptionally active U2OS nuclear extracts (300 ng/µL final) for 2hs at 30° C in 20 mM Hepes (pH 7.9), 10 mM Tris (pH7.9), 8 mM $(NH_4)_2SO_4$, 2 % PEG 6000, 8 mM $MgCl_2$, 10% Glycerol, 0.1 mM EDTA, 5 mM DTT, 50 mM KCl, 0.25 mM each rNTP except UTP, 5 nM UTP, [alpha-32P]UTP (10 µCi per reaction). T1 RNase was added to the reaction 30 min before stopping reaction with 150 µL stop buffer (1 mg/mL yeast tRNA, 10 mM EDTA, 100 mM Na2CO3, 0.2 % SDS). RNA was purified by phenol-chloroform extraction and precipitated with ethanol. *In vitro* transcription products were analyzed on a 6% denaturing PAAG.

Electrophoretic mobility shift assays (EMSA) RNA and DNA EMSA were performed as previously described[3]. Poly (dI-dC)/Poly (dI-dC) and tRNA were used as non-specific and control competitors. RNA probe corresponds to (-39 to +104) region and DNA probe corresponds to (-174 to +104) region in relation to the major promoter start site.

RNAi The knockdown of minor transcript was done by siRNA with the following sequence: AGCGGUUUUGAGCCGAUUCtt (antisense for ncRNA). A Silencer Control siRNA (Ambion, #4611) of scrambled sequence that does not target any human gene product was used as a negative control. Cells were co-transfected in a 6 well plate with siRNA (30nM) complexed with siPORTTM NeoFXTM Transfection agent (Ambion). After 72 hrs, the cells were harvested to test the efficiency of down regulation by RT-PCR and occupancy of TFIIB by ChIP.

Chromatin immunoprecipitation (ChIP) ChIP assay was performed as previously described[4]. Co-precipitated DNA was quantified by real-time PCR using QuantiTeck SYBR Green PCR kit (Qiagen) and primers (AGAGGATGGGGCCAGACTT) and (AGAATCCGGGCAGAAATCAG). The size of the amplified fragment is 120 bp.

ChIP on transfected DNA was performed in general as described above with some modifications. Fixed cells were ressuspended in 500 µL RIP lysis buffer (see RIP method). Sample was diluted to 1 mL and adjusted to 0.5% triton, 25 mM $MgCl_2$, 5 mM $CaCl_2$ and chromatin was digested with Pst I (1000 units at 37 °C for 2hs). Digesting was stopped with 20 mM EDTA. Chromatin was diluted 5 times with ChIP dilution buffer[4]. ChIP procedure was continued as describe above. Co-precipitated DNA was quantified by real-time PCR using QuantiTeck SYBR Green PCR kit (Qiagen) and the following primers: ATCCACTTAACCAATTCCTCAA and ACCTCAAAGTCATGGTTAGGG. The size of the amplified fragment is 150 bp.

Quantitative Immunoprecipitation (ChIP) from in vitro system DNA template (400 ng) was incubated with U2OS cell extracts (6 µg) in the same conditions as used for *in vitro* transcription in the presence or absence of NTP for 1 hour in 20 µL. DNA-protein complex was fixed with formaldehyde (1 % final concentration) for 10 min. Cross linking reaction was stopped by adding glycine pH 8.3 (0.4 M final concentration). Immunoprecipitation and DNA isolation were performed according to the ChIP procedure (see above). Co-precipitated DNA was analyzed by real-time PCR. In the case of the template containing major and minor promoters Pst I restriction was performed overnight at 37° C.

RNA Immunoprecipitation (RIP) RIP essay was performed with modifications of the procedure as described[5]. $4x10^7$ cells were crosslinked with 1% formaldehyde for 10 min and harvested in 10 ml of PBS. Crosslinking reaction was stopped by adding glycine to 330 mM final concentration. Cells were re-suspended in 500 µL RIP lysis buffer (50 mM HEPES pH7.5, 1mM EDTA, 1% triton) and sonicated 10 times for 10 sec. Sample was diluted and adjusted to 0.5% triton, 25 mM $MgCl_2$, 5 mM $CaCl_2$ and DNA was digested with DNase I RNase Free (30 units) at 37 °C for 15 min. Digesting was stopped with 20 mM EDTA. Sample was pre-cleared with protein G beads for 2 hrs at 4 °C and followed by addition of 10 µg of antibodies. 20 µl of protein G beads were added after 2 hrs of incubation and left for another 1 h at 4°C. Beads were washed once with 1 ml binding buffer (50 mM HEPES/0.5 % triton/25 mM $MgCl_2$/5 mM $CaCl_2$/20 mM EDTA), once with FA500 (50 mM HEPES/500 mM NaCL/1 mM EDTA/1% triton/0.1 % Na deoxycholate), once time with LiCl buffer (10 mM Tris/250 mM LiCl/1 % triton/0.5 % Na deoxycholate/1 mM EDTA) and once with TES (10 mM Tris/10 mM NaCL/1 mM EDTA). Immunoprecipitates were eluted with 75 µl RIP elution buffer (100 mM Tris pH 7.8/10 mM EDTA/1 % SDS). NaCl was adjusted to 200 mM and the samples were treated with 20µg of proteinase K for 1 h at 42 °C and 1 h at 65 °C. RNA was extracted with TRIzol solution (Invitrogen) and analyzed by RT PCR with the primers (ACCTGGTCGGCTGCACCT) and (TTGCCCTGCCATGTCTCG) .

H-form formation H-form (triplex complex) formation was performed with (i) DNA duplex running from -39 to +104 and ncRNA corresponding to the same positions or (ii) between duplex formed with deoxyoligonucletides A44 (ACAAATGGGGACGAGGGGGGCGGGGCGGCC) and A45 (GGCCGCCCCGCCCCCCTCGTCCCCATTTGT) and ribioligonucleotides of the same sequence as A44. In case of (i), 2 nM 32P-labeled dsDNA was incubated for 2 hrs at room temperature with increasing concentration of DHFR ncRNA or U1. Samples were resolved by 6% PAGE under non-denaturing conditions. In case of (ii), triple helix formation was performed as described earlier[6]. 2 nM of the 32P-labeled DNA duplex was incubated in TBM buffer (90 mM Tris, 90 mM borate, 10 mM MgCl2) for 2 hrs at in presence of increasing concentration of the ribooligonucleotide. Samples were resolved by 12% PAGE under non-denaturing conditions.

Inducible transcription The DHFR minor promoter was replaced with the human MeThio promoter[7] in a construct shown in (fig. 3c). The plasmid was transfected into U2OS cells using FuGENE 6 (Roche), 36 hours after transfection CdCl2 was added to cells to the final concentration 5 µM. 2 hours after induction cells were fixed with 1% formaldehyde and tested in ChIP assay.

Tet-dependent inducible transcription was performed using T-REx system (Invitrogen) with the constructs pcDNA4/TO and pcDNA6/TO, as described[8]. The minor DHFR promoter was replaced by CMV promoter under the control of Tet repressor by cloning promoter region the DHFR gene from -430 to +156, followed by G-less cassette into pcDNA4/TO, as shown on fig. 3d. Additional insertions included the pcDNA6/TR (Invitrogen) fragment 4465-6420 for extended elongation of transcription and the pcDNA6/TR (Invitrogen) fragment 4100 to 4464, containing SV40 polyA and termination sequence.

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