Chromatin dynamics at the hTERT promoter during transcriptional activation and repression by c-Myc and Mnt in Xenopus leavis oocytes

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The transcription factors c-Myc and Mnt regulate gene expression through dimerization with Max and binding to E-boxes in target genes. While c-Myc activates gene expression via recruitment of histone modifying complexes, Mnt acts as a transcriptional repressor. Here, we used the Xenopus leavis oocyte system to address the effect of c-Myc and Mnt on transcription and chromatin remodeling over the E-box region in the human telomerase reverse transcriptase (hTERT) promoter. As expected we found elevated and decreased levels of hTERT transcription upon exogenously expressed c-Myc/Max and Mnt/Max, respectively. In addition, we confirmed binding of these heterodimers to both E-boxes already enriched with H3K9ac and H4K16ac. These chromatin marks were further enhanced upon c-Myc/Max binding followed by increased DNA accessibility in the E-box region. In contrast, Mnt/Max inhibited Myc-induced transcription and mediated repression through complete chromatin condensation and deacetylation of H3K9 and H4K16 across the E-box region. Importantly, Mnt was able to counteract c-Myc mediated activation even when expressed at low levels, suggesting Mnt to act as a strong repressor by closing the chromatin structure. Collectively our data demonstrate that the balance between c-Myc and Mnt activity determines the transcriptional outcome of the hTERT promoter by modulation of the chromatin architecture.

Introduction

Myc exerts its biological functions by regulating genes important for cellular processes such as cell growth and division, cell cycle progression, apoptosis, and differentiation. In human cancer, c-Myc is the most frequently deregulated oncogene [1]. The c-Myc protein as well as Mxd and Mnt belongs to the family of basic-helix-loop-helix-leucine-zipper (bHLHZip) transcription factors.

Abbreviations: Ac, acetylation; AU, arbitrary units; bHLHZip, basic helix loop helix leucine zipper; bp, basepair; ChIP, chromatin immunoprecipitation; DMS, dimethylsulfate; DNase I, deoxyrribonuclease I; dsDNA, double stranded DNA; EMSA, electrophoretic mobility shift assay; HAT, histone acetylase; HDAC, histone deacetylase; HEK293T, human embryonic kidney 293T; hTERT, human telomerase reverse transcriptase; ssDNA, single stranded DNA; WB, Western Blot.

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and binds specifically to E-box sequences, CACGTG, as heterodimers with Max [21]. c-Myc/Max complexes bind preferentially to promoters enriched for euchromatin marks such as H3K4me2, H3K4me3, H3K79me2, H3K8ac, H3K18ac and H3K27ac [3–6]. In addition, Myc was found in genomic regions containing CpG islands, which generally are associated with transcriptionally active promoters [7]. Myc was recently shown to accumulate in the promoter regions of active genes where it acts as an amplifier of expression rather than being an on-off specifier of gene activity [5,6]. However, the on-off switch might be controlled by c-Myc indirectly since c-Myc interacts with 10–15% of human promoters (reviewed in [8]).

The accessibility of the chromatin is crucial for gene expression. Nucleosomes, consisting of DNA wrapped around the histone octamer (histone H3, H4, H2A and H2B), are packed into higher order chromatin structures restricting protein binding to DNA. Thus, local modulation of DNA accessibility influences the transcriptional outcome [9]. Once c-Myc/Max is bound to the promoter additional histone modifications are induced by recruitment of various cofactors including histone acetyltransferases (HATs) Tip60 [10], GCN5/PCAF [11,12] and p300/CBP [13], HAT associated proteins such as Transactivation/transformation domain associated protein (TRRAP) [11,14], members of the SWI/SNF chromatin remodeling complex (SNF5 and BRG1) (reviewed in [15–17]), histone methyltransferase mixed-lineage leukemia 1 (MLL1) [18] and ATPase/helicase TIP48 and TIP49 [19,20].

Myc induced transactivation is antagonized by binding of Mxd/Max or Mnt/Max heterodimers to the same E-box sequence resulting in establishment of an inactive chromatin conformation through recruitment of mSin3 containing corepressor complexes possessing histone deacetylase (HDAC) activity [21,22]. HDACs remove acetyl groups from histones, leading to decreased histone acetylation, chromatin condensation and, hence, transcriptional repression [23].

We have previously analyzed the promoter of the c-Myc/Mxd/ Mnt target gene human telomerase reverse transcriptase (hTERT), and found a switch from c-Myc/Max to Mad1 (Mdx1)/Max binding during differentiation of human promyelocytic leukemia (HL60) cells resulting in decreased promoter histone acetylation followed by reduced hTERT expression [24]. In contrast to c-Myc and Mxd, which are expressed in proliferating and differentiating cells, respectively, Mnt is ubiquitously expressed and Mnt/Max complexes are present at the promoter during both cellular stages [24,25]. However, we found that Mnt mediated transcriptional repression is inhibited through phosphorylation of the protein in proliferating cells [25]. Furthermore, since Mnt has been suggested as a key regulator of the Myc network as well as a potential tumor suppressor (reviewed in [26]) we wanted to study how c-Myc and Mnt regulate gene expression by remodeling of the chromatin structure across the E-box region in the hTERT promoter. To this end, we reconstituted the promoter in the Xenopus laevis oocyte system [27]. Oocytes from the African clawed frog are large cells allowing cytoplasmic microinjection of in vitro synthesized c-Myc, Max or Mnt mRNAs(s) and nuclear injection of the corresponding expression vector(s) [28]. Reporter DNA containing the hTERT promoter is provided by injection into the oocyte nucleus where it undergoes chromatin assembly within a few hours. In this way, the interaction between newly assembled chromatin of the hTERT promoter and c-Myc/Max or Mnt/Max as well as chromatin dynamics during transcriptional activation/repression by c-Myc and Mnt can be analyzed at high precision.

Materials and methods

Ethics statement

Ethic permissions N61/09, #C10/10 and N21/12 were approved by the Stockholm regional ethical committee for animal research.

Xenopus oocyte microinjection and plasmids

Defolliculated stage VI Xenopus laevis oocytes were prepared by collagenase treatment [29]; microinjections of DNA and mRNA into the oocytes were performed as described previously [30]. The c-Myc, Max, Mnt cDNAs were cloned between the BamH1/Not I sites in the RN3P vector, used for mRNA production as described [31]. The plasmids were linearized with Acc65I and in vitro transcribed using the mMESSAGE mMACHINE kit (Ambion). Obtained mRNAs were purified on a spin column (MEGaclear, Ambion). Indicated amount of c-Myc, Max or Mnt mRNA were injected in 23 nl into the cytoplasm. 5 to 7 h after mRNA injection, 3 ng single stranded (ssDNA) or double stranded (dsDNA) of the hTERT promoter plasmids hTERT A (p1009, [32]), hTERT B (p330, [32]), hTERT C (pB7-255, [33]), or minM4tkLuc [34] in 18.4 nl were injected into the oocyte nucleus. For expression of the hTERT containing reporters as ssDNA, –1009/+360 bp fragment (hTERT A) or –330/+360 bp fragment (hTERT B) were cloned into M13-mp19 bacteriophage vector using KpnI and SacI. For some experiments DNA expression vectors for c-Myc (pSp-Myc), Max (pSp-Max), Mnt (pRc-Mnt1A) were used to express the corresponding protein. In this case the plasmids were co-injected into the nucleus together with the hTERT or minM4tkLuc reporter. Injected oocytes were incubated in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2,1 mM MgCl2, 1 mM Na2HPO4, 5 mM HEPES pH 7.8) containing 10 μg/ml gentamicin (Sigma-Aldrich) at 19 °C for 24 h before analysis (Fig. SI1A). Pools of 7–10 injected oocytes of each group were used and all experiments were performed in duplicates or triplicates.

Analysis of intracellular amounts of exogenously expressed proteins

Xenopus oocytes injected with c-Myc, Max, Mnt mRNA, or expression vectors for Mnt or Max were incubated in OR2 medium containing 3H-Lysine. 24 h post injection the oocyte nuclei were manually dissected in nuclear isolation buffer (20 mM Tris–HCl pH 7.5, 0.5 mM MgSO4, 140 mM KCl) and homogenized in 10 mM Tris–HCl pH 7.5 containing complete protease inhibitor cocktail (Roche). Protein extracts were separated in 8 or 10% SDS PAGE followed by Western blot or by autoradiography of the dried gels. Western blotting was performed as previously described [35]. Membranes were probed with anti-c-Myc antibody (N262), anti-Max (C17) or anti-Mnt (M132), all from Santa Cruz Biotechnology Inc, or anti-actin (A2103) from Sigma. HRP-conjugated anti-rabbit antibody (Amersham Biosciences) was used as secondary antibody. Membranes were developed by enhanced chemiluminescence (ECL, Amersham).
Cell culture and transient transfection

Human embryonic kidney (HEK293T) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) medium supplemented with 10% FBS and 1% Glutamine. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Luciferase reporter assay

Xenopus oocytes were microinjected with c-Myc, Max or Mnt mRNA or with Max and Mnt expression vectors together with the hTERT promoter DNA (hTERT A, B or C) or miniM4tkLuc. HEK293T cells were transiently transfected with pSP-Myc, pSP-Max, pSP-vector, pRc-Mnt1A or pRc-vector together with hTERT promoter (hTERT A, B or C) or miniM4tkLuc DNA. All transfections included pCMV-β-galactosidase as control for transfection efficiencies. Twenty-four hours post transfection or oocyte injection, cells were lysed in extraction buffer (25 mM Tris pH 7.8, 2 mM EDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100) and analyzed for luciferase activity in Analysis buffer (25 mM Glycylglycin, 15 mM MgSO4, 5 mM ATP) containing 0.2 mM Luciferin using a luminometer. Luciferase activity in cells was normalized for differences in β-galactosidase levels as control for transfection efficiencies.

Chromatin immunoprecipitation (ChIP)

Pools of ten oocytes were microinjected with c-Myc/Max or Mnt/Max mRNA and hTERT A promoter DNA 24 h prior to crosslinking with 1% (v/v) formaldehyde for 10 min at ambient temperature. Next, oocytes were homogenized in 10 mM Tris–HCl pH 7.2, 0.1% SDS and complete protease inhibitor cocktail (Roche). Chromatin equivalent to 1 oocyte was used for immunoprecipitation in buffer 1 (0.1% Na-deoxycholate, 1% Triton-X-100, 2 mM EDTA, 10% glycerol, 1% Triton X-100) and analyzed for luciferase activity in Analysis buffer (25 mM Glycylglycin, 15 mM MgSO4, 5 mM ATP) containing 0.2 mM Luciferin using a luminometer. Luciferase activity in cells was normalized for differences in β-galactosidase levels in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol) containing 0.8 μg/μl ortho-nitrophenyl-β-galactosidase (ONPG). After incubation at 37 °C for appropriate time 0.3 M of NaCl was added and the β-galactosidase levels were measured in an ELISA reader at 420 nm.

DMS methylation protection assay and primer extension

DMS methylation protection assay was performed as described previously [27]. In brief, pools of 10 oocytes were treated with DMS in 500 μl OR2 medium containing 1.5 μl of DMS for 10 min at room temperature. After washing in OR2 solution oocytes were homogenized and treated overnight with proteinase K (100 μg/ml) in 1% SDS and 10 mM EDTA. After DNA purification the methylation pattern was developed by primer extension using Deep vent (exo-) polymerase (New England Biolabs) and [32P]-labeled primer (5′-AGACCTTCCGGTATGGCT-3′) as described previously [27]. Products of linear amplification were separated in 6% denaturing PAGE followed by analysis of dried gel with Fuji Bio-imaging analyzer BAS-2500 using Image Gauge V3.3 software.

DNase I hypersensitivity assay

Oocytes injected with hTERT A or B promoter DNA and c-Myc/Max or Mnt/Max RNA or DNA expression vectors was digested with DNase I as described previously [30]. In brief, pools of twenty oocytes were homogenized using Dounce homogenizer in 10 mM Tris–Cl (pH 8.0) 50 mM NaCl, 1 mM DTT, 5 mM MgCl2, and 5% glycerol. The homogenate was divided into four aliquots and DNase I was added (2, 4, 8 and 16 units per tube). Following incubation at 15 °C for 5 min, the reaction was stopped by addition of SDS to 1%, EDTA to 10 mM and Proteinase K to 100 μg/ml. After overnight incubation at 37 °C the DNA was purified via phenol/chloroform extractions and ethanol precipitation. Thereafter, the DNA was cleaved with KpnI/Bsu36I (hTERT A) and BstEI/XmnI (hTERT B) in the presence of RNase A (30 μg/ml), resolved in 1.5% agarose gel, vacuum transferred and hybridized with [32P]-labeled probe. The probe was prepared by purification of the BglII/EcoRI digested fragment cleaved from hTERT plasmid DNA.

Results

Changes in histone H3K9 and H4K16 acetylation status across the E-box region of the hTERT promoter upon c-Myc/Max or Mnt/Max binding

In order to understand in more detail how c-Myc and Mnt proteins regulate gene expression we have studied in vivo changes in chromatin structure across the E-box region in the hTERT gene reconstituted in Xenopus oocytes (Fig. S1A). First, DNA expression vectors or in vitro synthesized mRNAs encoding c-Myc, Max or Mnt were injected into the oocyte nuclei or cytoplasm, respectively (Fig. S1A). The expression of the corresponding proteins was detected either via 14C-Lysine incorporation followed by detection by radio autography and/or phosphoimaging or by Western blot analysis of the manually isolated oocyte nuclei or whole oocytes (Fig. S1B–E). Next, we used electrophotoretic mobility shift assay (EMSA) to show that exogenously expressed c-Myc (Fig. S2A) and Mnt (Fig. S2B) form heterodimers with introduced Max and that these complexes are able to bind specifically to the E-box sequence (CACGTCG) (Fig. S2A and B) in vitro. Interestingly, when injecting mRNA encoding Max alone we identified a novel Max-containing complex (X/Max, Fig. S2A), which may contain Xenopus Msc [36], Mxd or Mnt proteins [37]. Due to the mobility
of the band in the gel we reasoned that it most likely contained Myc and to test this we used several different antibodies against c-Myc and MYCN but none of them identified the protein interacting with Max (data not shown). Thus Xenopus specific antibodies would be needed to clarify the identity of this band.

Using chromatin immunoprecipitation (ChIP) assays we demonstrated in vivo binding of c-Myc/Max and Mnt/Max to the 5′ and the 3′ E-box in the \( hTERT \) promoter (Fig. 1A and B). We also performed analysis of histone modifications across the E-box region at the promoter co-occurring with c-Myc/Max or Mnt/Max binding and found a characteristic pattern indicative of transcriptional activation i.e. acetylation of H3K9 and H4K16 upon c-Myc/Max binding. Importantly, acetylation of histone H3K9 and H4K16 was detected in oocytes injected with the \( hTERT \) reporter alone but the levels were enhanced in oocytes expressing c-Myc/Max, suggesting binding to already active chromatin. In contrast, exogenous expression of Mnt/Max led to deacetylation of histone H3K9 and H4K16 (Fig. 1B).

![Fig. 1](image-url)

**Fig. 1** – In vivo binding of exogenously expressed c-Myc/Max and Mnt/Max and histone acetylation status at the \( hTERT \) promoter. (A) Overview of the \( hTERT \) A promoter construct showing the two E-boxes and the \( hTERT \) E-box primers (indicated by arrows) used in the ChIP assays. (B) Crosslinked chromatin from oocytes injected with 5.4 ng c-Myc, 0.7 ng Max or 3.1 ng Mnt mRNA in combination with 3 ng \( hTERT \) A promoter ssDNA was immunoprecipitated with antibodies against c-Myc, Max, Mnt, IgG, H4K16ac and H3K9ac. PCR primers covering the 5′ E-box or the 3′ E-box in the \( hTERT \) promoter as well as control primers (vector) were used. PCR amplifications of DNA from total chromatin were used as input. The data shown are representative from two or three independent experiments.
Transcriptional activation and repression by c-Myc and Mnt, respectively

In order to analyze the transcriptional response in oocytes expressing c-Myc/Max and Mnt/Max we used luciferase reporter assays (Fig. 2, Fig. S3A and B) and S1 nuclease protection analysis (Fig. S3C). Three hTERT promoter constructs, containing different lengths of the promoter fused to the luciferase gene were used (Fig. 2A). A titration of the amount of injected c-Myc mRNA and Mnt expression vector (Fig. S3B) revealed a 2–2.5 fold increase in luciferase activity at the optimum concentration of c-Myc and an almost complete transcriptional repression by Mnt/Max in Xenopus oocytes (Fig. 2B). Similar results were observed in HEK293T cells (Fig. 2C). In addition, we also analyzed the levels of hTERT mRNA in the context of c-Myc/Max or Mnt/Max expression by S1 nuclease protection assay (Fig. S3C). By co-expression with c-Myc/Max the levels of hTERT transcripts in the oocytes increased with 1.5 fold whereas Mnt/Max expression repressed the levels with 50% (Fig. S3C). Interestingly, co-injection of Myc mRNA together with increasing amounts of Mnt DNA expression vector resulted in transcriptional repression even though Mnt was expressed at low levels (Fig. 2D). When the amount of Mnt expression vector injected into the oocyte nucleus was diluted down to 0.01 ng, the basal hTERT transcription was no longer repressed and c-Myc/Max was able to activate transcription by almost two-fold (Fig. 2D). Furthermore, we showed transcriptional activation and repression of an artificial promoter (minM4tkLuc) containing four E-boxes fused with the luciferase gene in oocytes expressing introduced c-Myc and Mnt/Max, respectively (Fig. S3A and B). Taking together, our data show that expression of c-Myc/Max or Mnt/Max in the Xenopus oocyte model resulted in activation and repression of hTERT driven transcription, respectively, and that the balance between these two heterodimeric complexes determines the transcriptional outcome.

Sequence specific analysis of c-Myc/Max and Mnt/Max binding to the hTERT promoter

To further characterize in vivo binding of c-Myc/Max and Mnt/Max to the hTERT promoter we used dimethyl sulfate (DMS) methylation protection assay [27]. This method allows detection of sequence specific DNA-protein interactions in the living cell via methylation at the N7 position of guanines in the major groove of DNA. Methylated guanines are then converted to DNA strand breaks [38], which are developed by 32P-labeled primer extension. Thus, protein-DNA interactions are revealed as changes in DMS reactivity caused by bound proteins, i.e. by protection from methylation at these bases. Upon expression of c-Myc/Max we observed two protected guanine residues located within the 3′CACGTG palindrome. In addition, we observed an enhanced signal

Fig. 2 – Transcriptional response of the hTERT promoter induced by expressed c-Myc/Max and Mnt/Max. (A) An overview of the hTERT promoter constructs (hTERT A, B and C) used in the Luciferase reporter assays. hTERT A and B are in the pGL2-Enhancer vector and hTERT C in the pGL3-Basic vector, all three containing the Luciferase reporter gene. (B-C) Luciferase activity was measured after injection of 3 ng promoter construct (hTERT A, hTERT B, hTERT C) alone or in combination with 2.2 ng c-Myc mRNA (hTERT A and B) or 4.3 ng c-Myc mRNA (hTERT C), 8 ng pRc-Mnt 1A DNA, and 2 ng pSp-Max DNA in Xenopus oocytes (B) or 0.2 μg promoter construct (hTERT A, hTERT B, hTERT C) alone or in combination with 1.2 μg pSp-Myc or 1.2 μg pRc-Mnt 1A in HEK293T cells (C). (D) Luciferase activity and protein expression were measured in oocytes injected with a titration of pRc-Mnt 1A DNA (8, 4, 2, 1, 0.5, 0.1 and 0.01 ng), 2 ng pSp-Max, 3 ng c-Myc mRNA and 3 ng of the hTERT B promoter construct. Relative luciferase activity was presented as fold change in arbitrary units. Error bars indicate standard deviations. Data shown are from two independent experiments performed in triplicates.
for the band corresponding to the guanine located one bp outside of the consensus sequence (Fig. 3A, lanes 5, 6; scans to the left and graphs to the right). This finding indicates increased protein binding at the E-box, which is consistent with previous observations for other E-box containing genes [39]. Collectively, this argues for specific c-Myc/Max binding to the 3′ E-box in the hTERT promoter. However due to technical difficulties with the very GC-rich sequence we were not able to visualize the binding pattern across the 5′ E-box. Importantly, we noted that the methylation protection pattern in oocytes injected with the hTERT promoter together (Fig. 3A, lanes 5, 6) or without c-Myc/Max (Fig. 3A, lanes 3, 4) differed from that of naked DNA (Fig. 3A, lanes 1, 2) not only in the region of the 3′ E-box but also in other places, thus indicating binding at additional transcription factor sites (E2F, GC-box and others) in the promoter (Fig. 3A).

In contrast, E-box guanines were not protected from methylation in oocytes expressing Mnt/Max (Fig. 3A, lanes 7, 8). Moreover, the pattern of methylation protection pattern in these oocytes differed substantially from the one generated in oocytes injected with the hTERT DNA reporter alone (Fig. 3A, compare lanes 3, 4 and 7, 8) and overall looked very similar to that of naked DNA used as control (Fig. 3A, compare lanes 1, 2 and 7, 8). Together this suggests that Mnt/Max binding (Fig. 1B) generates a chromatin structure, which is not bound by other transcription factors. Thus, Mnt/Max mediates transcriptional repression by complete chromatin condensation of the hTERT core promoter, and thereby restricts the interaction of other transcription factors with the DNA (Fig. 3A). This further supports the notion of Mnt to act as a strong transcriptional repressor. Interestingly, we observed methylation protection of all four E-boxes in the artificial minM4tkLuc promoter in oocytes.

Fig. 3 - Sequence specific analysis of c-Myc/Max or Mnt/Max binding. (A) and (B) Characterization of c-Myc/Max and Mnt/Max binding by DMS methylation protection assay and primer extension of oocytes injected with 5.4/0.7 ng c-Myc/Max or 3.1/0.7 ng Mnt/Max mRNA in combination with 3 ng of the hTERT A promoter (A) or with 3 ng of minM4tkLuc construct (B). Naked hTERT (A) or minM4tkLuc (B) DNA were used as controls (DNA ctrl). A schematic overview of the hTERT promoter construct used is shown to left (A). The intensity of the bands was quantified by Fuji Bio-Imaging analyzer BAS-2500 using the Image Gauge V3.3 software and shown as scans next to the gel. Methylation protection of the guanines in the 3′ hTERT E-box (A, upper right) and in the E-boxes of the synthetic minM4tkLuc construct (B) are indicated with open circles. The hyper-methylated guanine positioned 1 bp downstream of the 3′ E-box (A, lower right) and the hyper-methylated guanines downstream of the E-boxes in the synthetic minM4tkLuc construct (B) are indicated with closed circles. Methylation protection/hypermethylation of indicated bands was quantified and related to the average of the reference bands (black triangles), setting the naked DNA to 100% methylation. The data shown are representative from two independent experiments.
expressing either c-Myc/Max (Fig. 3B, lane 4, 5) or Mnt/Max (Fig. 3B, lane 6, 7) thus suggesting that Mnt is not repressing the minM4tkLuc chromatin in the same manner as it does at the hTERT promoter.

**c-Myc/Max binding induces an open chromatin structure within the E-box region**

Next, we addressed the DNA accessibility of the chromatin across the E-box region at the hTERT promoter in oocytes expressing c-Myc/Max or Mnt/Max by DNase I hypersensitivity assay. DNase I rapidly digests open chromatin regions, and thereby identifies sites where chromatin remodeling takes place. Upon injection of the hTERT A or B reporter alone, DNase I hypersensitivity was observed just down stream of the 5' E-box as well as in the area between the two E-boxes (Fig. 4A, lane 1–4; and data not shown). Expression of c-Myc/Max resulted in enhanced DNA accessibility of this region (Fig. 4A, lane 5–8; and data not shown) compared to oocytes expressing the hTERT reporter alone (Fig. 4A, lane 1–4; and data not shown).

![DNA Accessibility](image)

**Fig. 4** – Increased DNA accessibility in the E-box region of the hTERT promoter upon c-Myc/Max binding. (A) DNA from Xenopus oocytes injected with 3 ng c-Myc or 8 ng Mnt and 2 ng Max DNA together with 3 ng hTERT B promoter DNA was digested with increasing concentrations of DNase I and hypersensitive sites were identified by Southern Blot and indirect end-labeling. hTERT promoter DNA digested with Kpn I and partially with Pml I (Pml I/Kpn I ctrl) was used to localize the E-boxes within the promoter. Quantifications of the bands are shown in the scans to the right. The scans were adjusted according to the intensity of the band corresponding to undigested DNA. Data are representative from three independent experiments. (B) Luciferase activity was measured in extracts from the pool of oocytes used for DNase I digestion and presented as fold change.
The Myc oncoproteins are deregulated in the majority of human cancers [1]. It has been shown that Myc/Max heterodimers interact with 10–15% of human promoters and target already active or potentially active, i.e., preset, chromatin by binding preferentially to promoters carrying histone H3K9 and H3K18 acetylation marks as well as di- and tri-methylations of H3K4 and di-methylation of H3K79 [3–8]. In this study, we have used the Xenopus oocyte model, which allows in vivo chromatin and transcriptional studies of a reconstituted promoter at high precision. As c-Myc has been suggested to be important for regulating hTERT transcription during immortalization [32,40–43] we wanted to investigate how c-Myc regulates gene expression at the level of chromatin remodeling. We showed c-Myc/Max binding to both E-boxes in the hTERT promoter, which were already enriched for acetylated histones H3K9 and H4K16. However, the basal levels of H3K9 and H4K16 acetylation observed in the oocytes injected with the hTERT reporter alone might be due to binding of endogenous Xenopus Myc. In addition, we observed a modest 2–2.5 fold activation of hTERT transcription by ectopically expressed c-Myc (Fig. 2, S3), which supports the role of Mnt in fine-tuning of gene expression. This could be due to recruitment of histone modifying complexes since we showed increased levels of H3K9ac and H4K16ac (Fig. 1B) upon c-Myc/Max binding. These factors in turn locally hyperacetylate histones resulting in disruption of the interaction between neighboring nucleosomes and, thus, opens the chromatin fiber [10,13,44–46]. In support, we demonstrated increased DNA accessibility between the E-boxes and at both the 5′ and 3′ E-boxes upon c-Myc/Max binding arguing for formation of a more open chromatin structure (Fig. 4A). The appearance of a major DNase I hypersensitive site located near the transcription start site in the promoter has previously been shown upon transcriptional activation [46]. This site may correspond to the DNase I hypersensitive site that we identified close to the 3′ E-box (Fig. 4A). In addition, Wang et al., showed that the nucleosomal organization of the hTERT promoter was changed upon differentiation of proliferating HL60 cells. These authors showed that the nucleosomal-free regions, which were present in the core promoter in proliferating cells, disappeared when cells were induced to differentiate and transcription was repressed [47]. Our data, showing differences in DNase I hypersensitivity as well as in the levels of histone acetylation across the E-boxes suggests differences in nucleosomal organization upon c-Myc/Max or Mnt/Max binding, respectively.

The hTERT gene is repressed in human somatic cells but reactivated upon immortalization (reviewed in [48]). This has been shown to be possible by for example inhibition of HDACs [24,46,49]. Here, we demonstrate that Mnt induces de-acetylation of histone H3K9 and H4K16 across the entire E-box region (Fig. 1B) and represses transcription of the hTERT promoter upon binding to both E-boxes (Fig. 1B, Fig. 253). Our data suggests Mnt to act as a strong transcriptional repressor since it inhibited c-Myc-induced transcriptional activation even when expressed at low levels. In addition, Mnt/Max completely repressed hTERT transcription induced by endogenous factors (Fig. 2). We also showed that Mnt/Max binding resulted in complete condensation of the hTERT core promoter chromatin (Fig. 3A). In contrast, we observed methylation protection of the guanines in all four E-boxes in the minM4tkLuc construct upon Mnt/Max binding (Fig. 3B). This suggests that Mnt does not direct the chromatin structure of the artificial promoter in the same manner as it does with the hTERT promoter. Specific chromatin folding characteristic for the artificial promoter and/or lack of binding partners/co-repressors, which are present in the hTERT promoter could be possible explanations. The fact that binding of Mnt/Max caused DNase I hypersensitivity in the E-box region (Fig. 4A), suggest a similar mechanism of gene silencing as has been reported for the Groucho (Gro/TLE/Grg) co-repressors [50]. Recruitment of Groucho by transcription factors was found to cause higher order condensed complexes of polynucleosome arrays, which surprisingly are in an open, exposed configuration accessible to DNase I action [50]. Thus, we suggest that Mnt/Max might repress transcription in a similar manner.

Taken together, our data show that c-Myc/Max binds to open/active chromatin region and contributes to the fine-tuning of gene expression by inducing local hyperacetylation of histone H3K9 and H4K16 and increased DNA accessibility of the hTERT core promoter. In contrast, Mnt/Max binding deacetylates H3K9 and H4K16, and represses transcription by complete condensation of the chromatin structure across the E-box region within the hTERT promoter. Thus, our findings strengthen the role of Mnt as a key regulator of the Myc/Max/Mxd/Mnt network.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.07.004.

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