

c-MYC coordinately regulates ribosomal gene chromatin remodeling and Pol I availability during granulocyte differentiation

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ABSTRACT

Loss of c-MYC is required for downregulation of ribosomal RNA (rRNA) gene (rDNA) transcription by RNA Polymerase I (Pol I) during granulocyte differentiation. Here, we demonstrate a robust reduction of Pol I loading onto rDNA that along with a depletion of the MYC target gene upstream binding factor (UBF) and a switch from epigenetically active to silent rDNA accompanies this MYC reduction. We hypothesized that MYC may coordinate these mechanisms via direct regulation of multiple components of the Pol I transcription apparatus. Using gene expression arrays we identified a 'regulon' of Pol I factors that are both downregulated during differentiation and reinduced in differentiated granulocytes upon activation of the *MYC-ER* transgene. This regulon includes the novel c-MYC target genes *RRN3* and *POLR1B*. Although enforced MYC expression during granulocyte differentiation was sufficient to increase the number of active rRNA genes, its activation in terminally differentiated cells did not alter the active to inactive gene ratio despite increased rDNA transcription. Thus, c-MYC dynamically controls rDNA transcription during granulocytic differentiation through the orchestrated transcriptional regulation of core Pol I factors and

epigenetic modulation of number of active rRNA genes.

INTRODUCTION

The complexity underlying the ability of the oncogene and transcriptional regulator MYC to regulate growth stems from its role in controlling ribosome biogenesis via transcriptional mechanisms that include all three RNA polymerases (I, II and III) (1,2). Central to c-MYC's role in growth is its ability to regulate expression of the 45S ribosomal RNA (rRNA) precursor (45S rRNA), a key rate limiting step of ribosome biogenesis that requires transcription of a proportion of the approximately 200 copies of rRNA genes by RNA Polymerase I (Pol I) (3). Intriguingly over 50% of the rRNA genes are transcriptionally silent at any one time (4,5). Thus, theoretically ribosomal gene (rDNA) transcription rate per cell can be modulated by the rate of transcription per active rRNA gene and/or the relative proportion of genes that are epigenetically active or silent.

MYC has been implicated in controlling rDNA transcription rate per gene through transcriptional control of the Pol I transcription initiation upstream binding factor (UBF) and through direct interaction of c-MYC with the rDNA itself and rDNA associated factors in the nucleolus (6–9). Intriguingly, in addition to transcription initiation,

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we and others have shown that UBF also regulates active ribosomal gene chromatin (r-chromatin) (10–12). Specifically, through binding across the promoter and entire coding region of the rRNA genes, UBF maintains an open chromatin structure, most likely by displacing linker histone (H1) (12,13). As UBF is a direct transcriptional target of c-MYC these data suggest r-chromatin remodeling is partially under the control of c-MYC. However, the extent of MYC's influence on rDNA silencing is unknown and the mechanisms by which MYC regulates rDNA transcription during dynamic and biologically relevant processes such as differentiation are poorly understood.

MYC is a transcription factor belonging to a family of basic helix-loop-helix-zipper (bHLHZ) proteins that bind as heterodimers to specific DNA sequences including the canonical E-box sequence CACGTG located in target gene promoters and much study has focused on identifying, analyzing and integrating the extensive cohort of c-MYC target genes (14–17). Differentiation is a physiological process that requires precise regulation of MYC function and thus provides a context to study the assimilation of fundamental MYC-driven gene pathways and mechanisms including rDNA transcription (18). Terminal granulocyte differentiation (TGD) is the process whereby progenitor cells restricted to the granulocytic lineage differentiate into mature neutrophils. Defined by their characteristic cell morphology, there are distinct stages of TGD: proliferating myeloblasts mature into promyelocytes then myelocytes followed by post-mitotic metamyelocytes and finally polymorphonuclear neutrophils. In addition to exiting the cell cycle, cells undergoing TGD exhibit a significant loss in cellular mass which in model systems of granulocyte differentiation occurs in a c-MYC dependent manner as measured by several parameters including cell volume, rate of *de novo* protein synthesis and 45S expression (6,19). A prerequisite for TGD is downregulation of c-MYC expression and in fact enforced expression of c-MYC blocks differentiation in several systems (19–22). While c-MYC is an established regulator of ribosome biogenesis, limited studies have investigated MYC's role in Pol I transcriptional regulation during differentiation and much of our mechanistic understanding derives from studies carried out in static model systems such as fibroblasts (6–9,23). Interrogation of how multiple MYC-dependent mechanisms of growth control coalesce during a dynamic process such as differentiation is crucial for our understanding of MYC function in a disease setting.

Downregulation of MYC during granulocyte differentiation leads to loss of UBF expression that correlates with an increased proportion of silent rRNA genes and repression of rRNA synthesis (6,12). This suggests a model where MYC controls rDNA transcription rates during TGD through silencing of rRNA genes. However, our data in mouse fibroblasts and that in yeast experiments (24,25) demonstrate that manipulating the number of active rRNA genes does not necessarily lead to a proportional change in cellular rRNA synthesis output. Following UBF depletion in the fibroblast model, while the majority of rDNA genes were silenced, the rate of Pol I

transcription was maintained due to a compensatory increase in the amount of Pol I associated with the remaining active rDNA repeats (12). Thus, at least under some conditions, rDNA silencing actually enhances the efficiency of rDNA transcription by concentrating Pol I around fewer active genes. Therefore, during granulocyte differentiation, although rDNA silencing may occur concomitantly with the downregulation of Pol I transcription, it is unlikely to be sufficient to regulate rDNA transcription rates.

In light of this, we have examined possible mechanisms by which MYC regulates rDNA transcription during differentiation using an *in vitro* model of TGD, the MPRO (mouse promyelocyte) model (26). Through expression profiling we have discovered that MYC regulates almost the entire repertoire of Pol I subunits and associated transcription factors. We show that members of this gene set, hereafter referred to as a Pol I 'regulon', are direct MYC target genes. Thus, as cells undergo differentiation and MYC expression is switched off, expression of factors driving rDNA transcription are likewise downregulated. Conversely, concomitant with the upregulation of rDNA transcription, the regulon is re-induced in differentiated MPRO cells upon activation of the conditional MYC-ER fusion protein. These data suggest a model whereby downregulation of rDNA transcription in response to loss of MYC during differentiation is modulated largely through reducing the availability of Pol I and its associated transcription factors. This serves to repress Pol I transcription in two ways: reducing UBF expression leads to silencing of the rRNA genes; critically however, expression of Pol I and its associated factors must also be reduced to prevent the hyperactivation of the remaining active rDNA repeats. Thus, during TGD MYC functions to couple the relative proportion of active rRNA genes with availability of the Pol I transcription apparatus to ensure efficient regulation of rDNA transcription. Given the ubiquitous and essential nature of MYC and rDNA transcription, we propose that this coordinate regulation of active rRNA gene number and Pol I component levels by MYC is a fundamental mechanism for controlling rDNA transcription in mammalian cells.

MATERIALS AND METHODS

Cell culture and cell analyses

The MPRO cell line (26) was cultured [in DMEM containing 20% fetal bovine serum (FBS) and BHK-HM5 conditioned media] and differentiated (in DMEM containing 10% FBS and BHK-HM5 conditioned media) by the addition of 10^{-6} M retinoid X receptor (RXR)-specific agonist AGN194204 (hereafter referred to as AGN) (Allergan, Irvine, CA, USA) as described in detail in ref. (19). MPRO cells were transduced with the pBabe-MYC-ER retrovirus (27) and MYC-ER was activated by addition of 200 nM 4-hydroxytamoxifen (4-OHT) as previously described (19). For cell morphology 30 000 cells were cytopspun onto glass slides, air-dried, methanol fixed and stained with May-Grunwald Giemsa (MGG).

Cell volume was determined using a Beckman Coulter Z2 instrument.

Psoralen crosslinking assay

Cells were lysed in NP-40 lysis buffer and nuclei recovered and irradiated in the presence of 4,5, 8'-trimethylpsoralen (psoralen) (Sigma) with a 366-nm UV light box as described previously (12). Genomic DNA was isolated and digested with SalI, separated on a 0.9% agarose gel and alkaline Southern blotting was performed. Psoralen crosslinking was reversed and the membrane was hybridized to a purified ³²P-labelled rDNA probe (12). Finally, the membrane was visualized on a PhosphoImager and quantitated using ImageQuant (TLv2005.04) (both GE Healthcare).

Western blotting

Immunoblotting was performed using 10–100 µg total protein extracts lysed in sodium dodecyl sulfate (SDS) lysis buffer and separated by SDS-PAGE as described (19). For detection of the RRN3 protein, 2–5 × 10⁷ D0 and D4 wt MPRO cells were harvested and S100 extracts were prepared as previously described (28). Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes and analyzed using the antisera listed below and enhanced chemiluminescence. The antibodies used were: anti-c-MYC (sc-764), anti-ERα (sc-542) and anti-RRN3 (sc-11805, wtMPRO Western) (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Actin (691002; MP Biomedicals, Solon, OH, USA); anti-UBF1/2 and anti-POLR1B (128 kDa subunit) are in-house polyclonal rabbit antisera (29); the polyclonal rabbit anti-PAF53 and the polyclonal sheep anti-RRN3 (MYC-ER MPRO Western) antisera were kind gifts from Prof. Lawrence I. Rothblum, Oklahoma City, Oklahoma and Prof. Brian McStay, Galway, Ireland, respectively.

Quantitative chromatin immunoprecipitation assay

Quantitative chromatin immunoprecipitation (qChIP) assays were carried out as described previously (6). Crosslinking was achieved with 0.8% formaldehyde and assays were performed using ~1–5 × 10⁷ cells per immunoprecipitation (IP). ChIP antibodies used were: anti-c-MYC, anti-UBF1/2 and anti-POLR1B (used for western analysis); anti-trimethyl H3K4 (ab8580) and anti-H4 (ab31827) (both from Abcam, Cambridge, MA, USA); and anti-hyperacetylated H4 (06-946), anti-acetyl H3K9 (07-352) and anti-H3 (07-690) (all from Upstate, Millipore, Billerica, MA, USA). Control antisera for ChIPs were normal rabbit IgG (sc-2027; Santa Cruz) and in-house pre-immune rabbit serum. For all ChIPs, 5 µg of purified antibody or 10 µl of sera were used per IP. Samples were analyzed in triplicate by quantitative real-time PCR (qPCR) using the SYBR Green mix on the StepOnePlus™ real-time PCR system (Applied Biosystems). To calculate the percentage total DNA bound, qPCR analyses of unprecipitated input samples were used as reference for corresponding IPs. Final enrichment was determined by subtracting appropriate control IP values. Primer sequences used for rDNA ChIPs are

all previously published (12) except for the 28S and T2 amplicons that along with those from all other ChIP amplicons are listed in Supplementary Table S1. ChIP-CHOP assays were performed by digesting DNA isolated from ChIPs with HpaII prior to qPCR and the relative level of HpaII-resistance was calculated after normalization to mock-digested DNA (12).

RNA isolation and gene expression analysis by quantitative real-time RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen) and reverse transcribed by Superscript III (Invitrogen) using random hexamers (Promega). Real-time PCR was performed as for ChIP samples then quantitated using the delta-delta CT ($\Delta\Delta C_T$) method. Primer sequences are either previously published (19) or listed in Supplementary Table S1.

Gene expression profiling

Total RNA was extracted from samples derived from four biological replicates using TRIzol and further purified using the Qiagen RNeasy Mini kit (Qiagen). RNA quality was assessed using the Agilent 2100 Electrophoresis Bioanalyzer and for each array 5 µg of total RNA was used to prepare the target probe with the Affymetrix One-Cycle Target Labeling reagents (Affymetrix Inc., Santa Clara, CA, USA). Probes were hybridized to Affymetrix Mouse Genome 430 2.0 arrays according to the manufacturer's instructions. Using Bioconductor (30), the gene expression data was imported into the statistical software suite R and following quality assessment of the chips (31), the data was normalized and background corrected using the robust multi-array average (RMA) expression measure with the help of probe sequence (gcRMA) (32). The package *Affy* (33) was used to produce MAS5 calls and the Limma package (34) was then used to model the data and assess differential expression. For each probeset the average log₂-expression for each treatment was calculated and pairwise comparisons performed. Any probesets that had a log₂-fold-change greater than 1 (corresponding to a 2-fold change in expression level) and a false discovery rate (FDR) adjusted $P > 0.05$ (35) were considered differentially expressed. The expression array data are available at www.ebi.ac.uk/arrayexpress under accession numbers E-MEXP-2786 (wt MPRO) and E-MEXP-2794 (MYC-ER MPRO).

RESULTS

MYC levels equate with UBF binding to rDNA and active rRNA gene number during TGD

To investigate the relationship between MYC regulation of UBF and Pol I transcription during granulocyte differentiation we utilized the MPRO model system where the role of MYC in promoting cell growth and inhibiting differentiation is well characterized (6,19). Parental MPROs (wt MPROs) were examined at multiple stages of differentiation (Figure 1A) during which time expression of

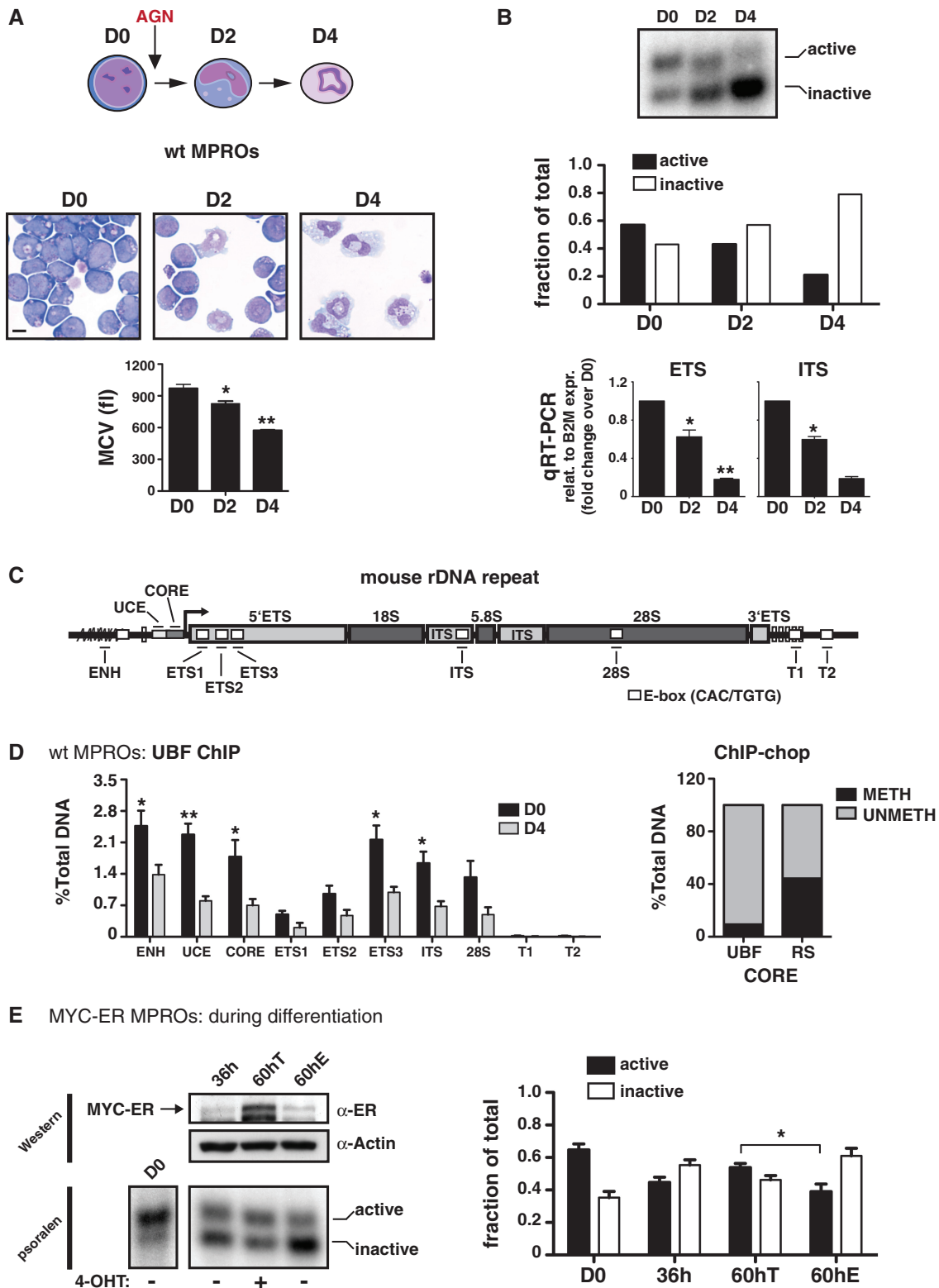


Figure 1. MYC regulates the epigenetic status of rRNA genes during granulocyte differentiation. (A) Top: schematic of wt MPRO cells induced to differentiate by addition of AGN. Proliferating cells (D0) are treated with AGN and re-characterized after 2 days (D2) and 4 days of differentiation (D4). Middle: morphology of wt MPROs at the stages described above. Bar = 15 μ m. Bottom: mean cell volume (MCV) in femtoliters (fl) for wt MPROs at the above stages. Results are the mean \pm SEM from four independent experiments. * $P < 0.05$ and ** $P < 1.0 \times 10^{-4}$ compared to D0. (B) Top: nuclei were extracted from wt MPROs at D0, D2 and D4 and subjected to psoralen crosslinking followed by Southern blot analysis of rDNA [representative analysis of previously published data (D0 and D4; 12)]. Middle: quantitated fractions of active and inactive ribosomal genes from the above Southern blot. Bottom: expression of the 45S rRNA precursor by qRT-PCR using primers directed against the 5' externally and internally transcribed spacers (ETS and ITS, respectively) and normalized to β -2 microglobulin (B2M) expression ($n = 4$). * $P < 0.005$ and ** $P < 5.0 \times 10^{-8}$ compared to D0. (C) Schematic of a single murine rDNA repeat with the positions of qPCR amplicons indicated: promoter

Continued

c-MYC and its target gene UBF are downregulated. MPROs undergo stereotypic TGD over 4 days upon treatment with the RXR-specific agonist AGN, progressing from promyelocytes prior to treatment (D0), to predominantly myelocytes/metamyelocytes at 2 days of AGN treatment (D2) and finally to differentiated neutrophils after 4 days of AGN (D4) (Figure 1A, top). Examination of the morphology of wt MPRO at D0, D2 and D4 revealed the changes expected during murine TGD: the stained cytoplasm appeared progressively paler as protein synthesis decreased and there was increasing chromatin condensation; by D4 segmenting nuclei had the distinctive ring-shaped appearance of murine neutrophils (Figure 1A, middle). Concurrently, wt MPROs displayed a typical reduction in cell size of 40% by D4 (Figure 1A, bottom).

Active rRNA gene number can be measured by relative crosslinking of psoralen, a DNA intercalating agent that incorporates into open chromatin associated with nascent rRNA transcripts but is inaccessible to silent genes associated with regularly spaced nucleosomes. Following psoralen crosslinking the active and inactive rRNA genes can be distinguished by a Southern blot assay based on the differing rates of migration of an rDNA SallI restriction fragment (36,37). In the MPRO model the number of active rRNA genes decreases during differentiation (Figure 1B, top and middle) correlating with a significant decrease in expression of 45S rRNA (Figure 1B, bottom) (6,12).

As regulation of active rRNA gene number occurs in a UBF-dependent manner, we next proceeded to thoroughly characterize UBF's association with the rDNA repeat during TGD. Utilizing primer sets for qPCR that amplify rDNA sequences representing functional sites and domains across the entire repeat, we performed qChIP analysis of UBF occupancy in D0 and D4 MPRO cells (Figure 1C and previously described in 12). Other than the minimal binding detected at the termination sequences (T1 and T2), UBF was present at considerable levels across the entire rDNA repeat in D0 cells and demonstrated an ~50% decrease in binding at all sites examined during differentiation (Figure 1D, left). High levels of absolute UBF occupancy are found at the proximal promoter UCE and CORE sites (Figure 1D, left) which span a key regulatory CpG dinucleotide at -133 from the transcription start site (TSS) that when methylated is minimally bound by UBF and renders the rRNA gene transcriptionally silent (12,38). The ChIP-CHOP assay (39), a method that determines the methylation status of DNA isolated by ChIP assay

via digestion with the methylation sensitive restriction enzyme HpaII followed by qPCR across the site, was performed on DNA from UBF ChIP assays in proliferating MPRO cells. ChIP-CHOP analysis revealed that UBF associates predominantly with active rRNA genes as determined by the unmethylated status at the -133 CpG dinucleotide of the majority of UBF bound rDNA (~90%) as compared to control rabbit serum bound rDNA (~55%) (Figure 1D, right). Therefore as absolute levels of UBF protein decrease during TGD, the amount of UBF associated with actively transcribed rDNA likewise decreases, ultimately coinciding with a reduction in the number of active genes.

In addition to regulating levels of UBF, the MYC protein has also been shown in several systems to directly interact with the rDNA repeat, via both binding E-boxes and protein-protein interactions with the SL-1 complex, and thus stimulate rRNA transcription (7-9). To assay MYC binding to the rDNA repeat in granulocytes, qChIP analysis in D0 and D4 MPRO cells was executed, assessing the same rDNA amplicons used for UBF qChIP (Figure 1C) which also correspond with canonical and non-canonical E-boxes. Notably, we detected modest although statistically significant enrichment of MYC across the rDNA repeat in proliferating granulocytes; absolute levels of MYC protein associated with the rDNA repeat were approximately 7-fold lower as compared with MYC binding at the UBF promoter, a positive control for a MYC-regulated gene (Supplementary Figure S1A).

We have observed a correlation between MYC-dependent loss of UBF expression during MPRO differentiation and its depletion from the rDNA repeat with an increase in the number of silent rRNA genes. To address the question of whether c-MYC is capable of driving changes in active rRNA gene number during TGD, we employed an MPRO system with inducible MYC activity. MPRO cells expressing the conditional *MYC-ER* gene (MYC-ER MPROs) were harvested at various stages of MPRO differentiation following activation of the MYC-ER fusion protein by addition of 4-OHT (27,40) and then subjected to psoralen crosslinking to determine the active versus inactive gene ratios. First, we activated MYC-ER by the addition of 4-OHT prior to initiating differentiation of MYC-ER MPROs with the retinoid agonist and then assayed psoralen ratios after 2 days of differentiation. By D2, 4-OHT treated MPROs undergoing a MYC-driven block to differentiation (19)

Figure 1. Continued

enhancer (ENH); proximal promoter sites of demonstrated factor binding including the upstream control element (UCE) and the core region (CORE); 5' ETS amplicons (ETS1, 2 and 3); 5' ITS; the stably expressed 28S rRNA; and transcription termination sequences (T1 and 2). E-boxes associated with amplicons are indicated with white boxes. (D) Left: qChIP analysis of UBF binding at rDNA sequences from (C). Analysis was performed on cells harvested prior to AGN treatment (D0) and at D4 of differentiation where the percent (%) total DNA value represents DNA enrichment with the anti-UBF antibody followed by subtraction of the control rabbit serum (RS) bound DNA (results from a minimum of three independent experiments). * $P < 0.05$ and ** $P < 0.005$ compared to D4. Right: ChIP-CHOP assay of UBF and RS ChIPs in D0 MPROs. DNA samples were both HpaII and mock digested prior to qRT-PCR analysis using the CORE primers to determine the relative fraction of HpaII-resistant, methylated rDNA (METH) with the remaining fraction designated as unmethylated (UNMETH) ($n = 1$). (E) MYC-ER MPROs (D0) were induced to differentiate for 36h (36h) before being treated with either vehicle control (EtOH) or 4-OHT for 24h (60hE and 60hT, respectively). Cells were harvested at indicated time points and protein lysates were analysed by Western blotting for expression of MYC-ER as compared to Actin (top left) while nuclei were analysed by psoralen crosslinking assay (bottom left) as described in (B). The relative fractions of active and inactive rRNA genes were quantitated and represented graphically (right) ($n = 3$). * $P < 0.05$.

maintained an active to inactive gene ratio similar to that of D0 cells whereas the partially differentiated D2 vehicle (EtOH) treated cells showed a majority of rDNA in the inactive state (Supplementary Figure S2A). This MYC-mediated failure to appropriately silence rRNA genes is likely to occur, at least in part, through MYC's maintenance of UBF expression.

We next treated cells with AGN to initiate differentiation before activating MYC-ER. After 60 h of differentiation, control cells had an active to inactive gene ratio of 40:60% (Figure 1E). In contrast, inclusion of 4-OHT in the media for the last 24 h of differentiation (added at 36 h) reversed the ratio of active to inactive genes from 45:55 at 36 h to 55:45% by 60 h (Figure 1E), consistent with observed higher rates of rDNA transcription (Supplementary Figure S2B). These data demonstrate that in the context of TGD, MYC is able to regulate silencing of rRNA genes, presumably indirectly through control of UBF levels.

Gene expression profiling during TGD identifies a set of MYC regulated core Pol I regulatory genes

In 3T3 fibroblasts, an increase in the number of silent rRNA genes due to loss of UBF leads to a compensatory increase in Pol I loading on the remaining active genes that results in a net maintenance of rDNA transcription rates (12). However, given that in the MPRO system 45S rRNA expression decreases significantly (Figure 1B), we examined the relative binding of Pol I per active rRNA gene in differentiating MPROs by qChIP using an antibody recognizing the second largest Pol I subunit (POLR1B) to determine if, in contrast to fibroblasts, TGD is associated with reduced loading of Pol I in addition to changes in the number of active genes (Figure 2A). Canvassing the entire repeat, ChIP assays revealed a substantial depletion of Pol I across the promoter enhancer (ENH) and transcribed region (ETS1-3, ITS and 28S) by D4 of MPRO differentiation, suggesting a reduction in Pol I complex loading that coincides with the decline in 45S rRNA levels (Figure 2A). This observed reduction in Pol I occupancy throughout the transcribed rDNA sequences is striking given that the qChIP results were normalized to ~50% decrease in active gene number (Figure 2A). Thus, in contrast to fibroblasts where Pol I loading on the remaining active genes increased in response to UBF depletion to maintain transcription output, during TGD the remaining active rRNA genes are also depleted of Pol I and in turn, rDNA transcription is downregulated.

In light of this significant decrease in Pol I apparatus association with rDNA during TGD, we hypothesized that in addition to UBF, MYC may coordinate the transcriptional downregulation of multiple core Pol I machinery components and thus render Pol I levels and its recruitment to rDNA limiting for transcription. To examine the entire repertoire of genes involved in Pol I transcription as well as those genes specific to Pol II and III, we conducted a global gene expression analysis where we generated the intersecting gene list from those genes with decreased expression during MPRO differentiation

and those with induced expression upon activation of MYC in MPROs. To achieve the latter, we utilized the MYC-ER MPROs that were differentiated (low endogenous MYC expression) and then harvested both prior to (D4) and following induction of MYC-ER expression by the addition for 24 h of either 4-OHT (D5T) or EtOH vehicle control (D5E) (Figure 2B) (6). Morphological analysis of MYC-ER MPROs showed the characteristic pale cytoplasm, condensed chromatin and ring-shaped nuclei of terminally differentiated murine granulocytes in both D4 and control D5E cells (Figure 2C, left and top right) whereas a considerable number of D5T cells appeared larger with an increased nuclear to cytoplasmic ratio as well as darker cytoplasmic staining consistent with an increased cytoplasmic ribosome content and upregulation of protein synthesis (Figure 2C, bottom right). We also confirmed that activation of MYC-ER in these differentiated cells leads to an approximately 2-fold induction of 45S expression demonstrating that activation of MYC-ER is sufficient to stimulate rDNA transcription (Figure 2D; 6).

Expression analysis was carried out for MPRO cell populations and of the 22 839 genes detected by the gene expression array, 1794 genes (7.9%) decreased in expression 2-fold or more from D0 to D4. Conversely, when MYC-ER was induced in D4 MPROs, 1897 genes (8.3%) increased in expression 2-fold or more from D5E to D5T. The Venn diagram of these two categories revealed that 1082 genes, or 60% of the genes that decreased in expression from D0 to D4 were re-induced with MYC-ER activation (D5T > D5E) (Figure 2E).

We next pre-defined a consensus set of annotated genes involved in Pol I transcription—termed a ‘regulon’—comprising genes described in the literature as being required for the formation of the Pol I pre-initiation complex and we similarly defined core factor regulons for both Pol II and Pol III for comparison with the Pol I regulon (Supplementary Figure S3) (41,42). To generate the regulons we also utilized Ingenuity Pathways Analysis that identifies gene networks based on a maintained database of known functional interactions, as an independent tool. The gene expression array data for Pol I regulon genes, MYC and its antagonist MAD1 (also known as MXD1) and the MYC target genes *ornithine decarboxylase* (ODC1) and *cyclin D2* (CCND2) are depicted in representational heatmaps (Figure 2F). Gene expression array signal intensities showed that expression of 64% of Pol I regulon genes decrease by >2-fold in wt MPROs and 79% of the Pol I regulon is likewise induced when comparing 4-OHT treated MYC-ER MPROs to EtOH treated cells (Supplementary Figure S3A). Notably, the 79% of Pol I regulon genes with >2-fold increased expression compares to 43% of Pol III regulon genes and only 14% of Pol II regulon genes (Supplementary Figure S3A–C).

Validation of gene expression data

Pol I transcription initiation requires a number of core factors: initially UBF forms a complex with selectivity factor 1 (SL-1, minimally comprised of TAF1A, B, C

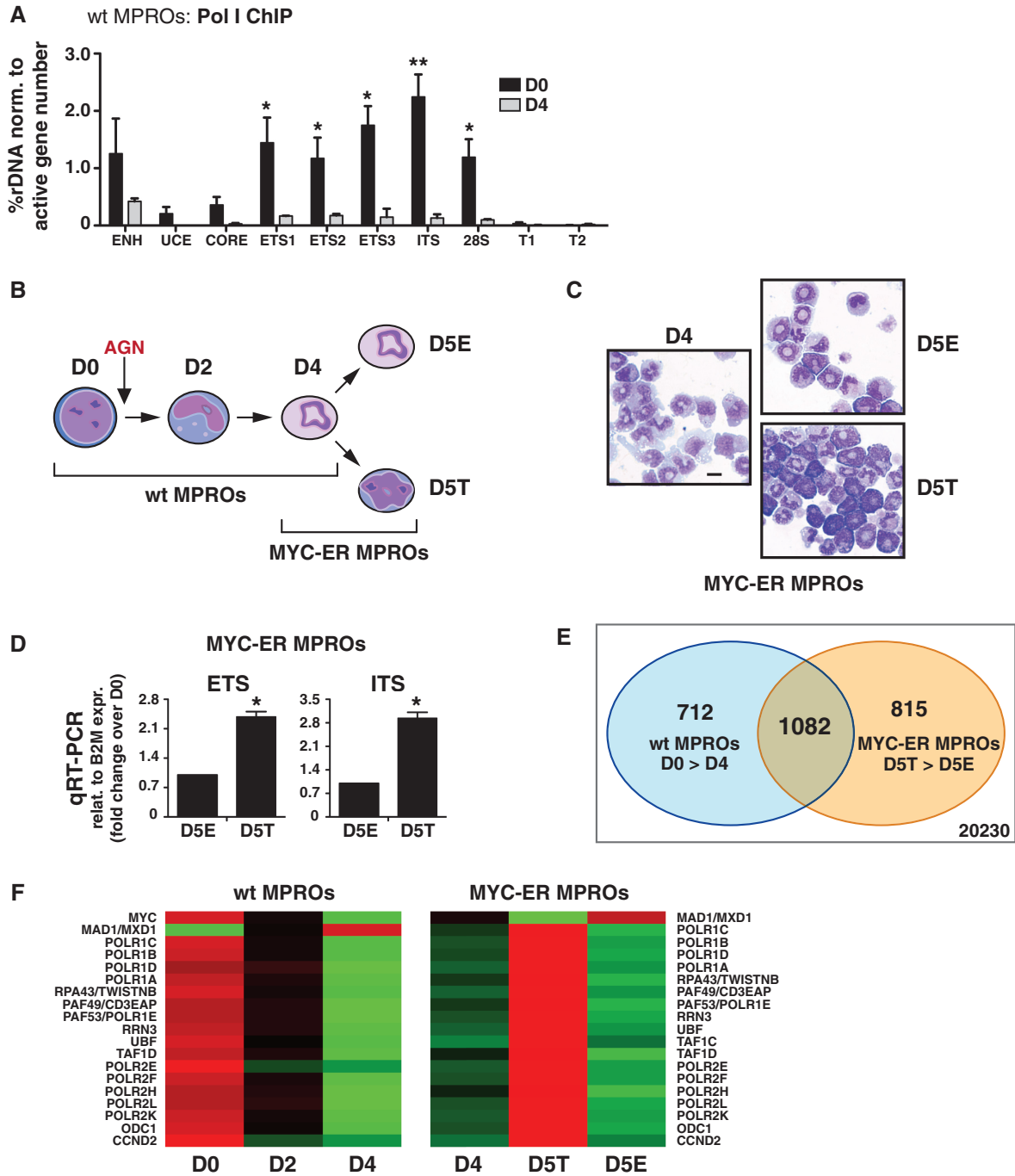


Figure 2. c-MYC regulates a Pol I regulon during MPRO differentiation and in differentiated MPROs with enforced MYC expression. (A) Pol I enrichment at the rDNA repeat normalized to the number of active genes. qChIP analysis of Pol I (anti-POLR1B, 128 kDa subunit) binding at rDNA sequences shown in Figure 1C was performed in D0 and D4 MPROs and analyzed by qPCR as described in Figure 1D. Results are the mean ± SEM from a minimum of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared to D4. The total percentage ChIP values were normalized to the average relative proportion of active genes for D0 (0.44) and D4 (0.19) MPROs as determined by psoralen crosslinking experiments described here (Figure 1B) and in our previously published report (12). (B) Schematic of wt MPRO proliferating cells (D0) induced to differentiate for 2 days (D2) and 4 days (D4). Differentiated MYC-ER MPROs (D4) were treated with EtOH vehicle or 4-OHT for 24h (D5E and D5T, respectively). (C) Morphology of MYC-ER MPRO cells treated as described in (B). Bar = 15µm. (D) Expression of the 45S rRNA precursor as described for Figure 1B ($n = 4$). * $P < 0.001$ compared to D5E. (E) Venn diagram illustrating the overlap between wt MPRO genes whose expression decreases 2-fold or more ($n = 4$, $P < 0.05$) from D0 to D4 and those MYC-ER MPRO genes whose expression is increased 2-fold or more ($n = 4$, $P < 0.05$) between D5T and D5E. (F) Heatmaps of transcripts from Pol I regulon genes and MYC target genes ODC1 and CCND2 displaying a significant change in expression [1.5-fold or more D0 > D4 ($n = 4$, $P < 5 \times 10^{-4}$) and D5T > D5E ($n = 4$, $P < 0.05$)] in MPRO populations in accordance with MYC expression. The MYC transcriptional antagonist MAD1/MXD1 shows an opposing expression pattern. The mean relative expression levels are represented by a color scale where red, high; black, mean; and green, low expression.

and D and TBP) at the rRNA gene promoter which in turn allows for recruitment of transcriptionally competent Pol I holoenzyme defined by the presence of RRN3 (also called TIF-1A) (5,41,42). The Pol I enzyme is a multisubunit complex including core subunits such as POLR1B and several Pol I associated factors such as PAF53 (42). To validate that the observed gene expression profiles of the Pol I regulon coincided with expression of MYC, changes in gene and protein expression of a subset of regulon factors—UBF, TAF1C, RRN3, PAF53 and POLR1B—were evaluated by quantitative real-time RT-PCR (qRT-PCR) and western blot analysis, respectively. qRT-PCR expression profiles of the subset revealed a tight correlation with their respective gene expression array signal intensities and mirrored the reduction of endogenous MYC while contrasting the increase in MAD1 expression during TGD (Figure 3A and B); likewise western blot analysis confirmed that decreases in expression also occurred at the protein level (Figure 3C). Activation of MYC transcriptional activity in differentiated MYC-ER MPROs treated with 4-OHT was confirmed by signal intensity and qRT-PCR data indicating induction of CCND2 (Figure 3D) while induction of the MYC-ER fusion protein was monitored by western blot analysis (Figure 3E). The subset of Pol I factors showed induced expression following the specific activation of MYC in D5T MPROs as determined by qRT-PCR, gene expression array and western analysis (POLR1B, UBF and RRN3) (Figure 3E and F). Expression profiles from both wt and MYC-ER MPROs did not show significant changes for TTF-1, a multifunctional protein involved in Pol I transcription termination and recently shown to have a role in the epigenetic regulation of r-chromatin but not considered to be a basal Pol I transcription initiation factor, illustrating the specificity of the regulon (Figure 3B and F) (43,44). Thus, the uniformity of data across all levels of gene expression—qRT-PCR, signal intensity and protein—strongly validate the gene expression array data sets and reinforce the co-regulatory expression patterns we observe between c-MYC and core Pol I transcription machinery.

UBF, RRN3 and POLR1B are direct transcriptional c-MYC target genes

Given the strong correlation between expression of core Pol I transcription factors and c-MYC in our model, we investigated whether in addition to UBF, other members of this regulon might also be direct MYC targets. Scanning 2 kb both up- and downstream of the TSS of select Pol I regulon genes for canonical (CACGTG) and non-canonical (CATGTG) E-boxes we identified a minimum of one E-box in the regulatory regions of all five genes (Figure 4A).

We then examined the role of c-MYC in the direct regulation of both RRN3 and POLR1B as representative of the active Pol I transcription complex. The RRN3 transcript was rapidly induced (within 3 h) in 4-OHT treated serum-starved NIH3T3 cells expressing the c-MYC-ER fusion protein; induction occurred in cells treated with cycloheximide, an inhibitor of protein synthesis, providing

evidence for direct regulation of RRN3 by MYC (Supplementary Figure S4A). To further address the question of direct, *in vivo* transcriptional regulation of these genes by c-MYC, we performed qChIP analysis to determine if MYC is physically associated with select RRN3 and POLR1B promoter E-boxes (Figure 4A). qChIP assays were carried out in D0 and D4 wt MPRO cells, providing the contrast of high and low levels of endogenous c-MYC protein respectively and overall, the degree of MYC binding at both the RRN3 and POLR1B promoters was similar if not greater to the levels of MYC found at the positive control UBF promoter E-box 1 in proliferating MPROs (Figure 4B). Of the E-boxes examined, the RRN3 promoter distal E-box (located 4 kb upstream of the TSS) and the POLR1B promoter E-box1 were bound by lower, non-significant levels of MYC protein in D0 cells indicating selectivity for MYC enrichment in the promoter regions (Figure 4B).

Several reports in recent years have begun to dissect what constitutes a genomic region of bona fide direct MYC interaction at a target gene (45–47). From these studies, one of the major chromatin marks to be associated with MYC binding at transcriptionally poised target promoters was trimethylated histone H3 at lysine 4 (H3K4me3) (45). To adjust for changes in nucleosomal density during TGD we carried out a qChIP analysis of total histones H3 and H4 at target gene E-boxes in D0 and D4 cells and subsequently normalized all histone mark ChIPs to respective total histone levels. Of note, we observed a general increase in overall histone levels at these sites, most likely due to chromatin condensation occurring as the cells differentiated (Supplementary Figure S5A). We next performed qChIP analysis using an antibody that recognizes H3K4me3 and detected high levels of enrichment specifically at E-boxes showing significant c-MYC occupancy, with H3K4me3 levels decreasing moderately during differentiation after normalization to H3 (Figure 4C, top). c-MYC recruits histone acetylases to target gene promoters which in part mediates activation of transcription via increased acetylation of multiple lysines on histones H3 and H4 (46,48). Using antibodies that recognize acetylated histone H4 (H4ac) and acetylated histone H3 at lysine 9 (H3K9ac) and again normalizing to total histone levels, qChIP assays detected greater association of acetylated histones at the MYC bound E-boxes in D0 than in D4 MPROs, the reduction of their occupancy during differentiation consistent with the parallel loss of MYC binding (Figure 4C, middle and bottom). Taken together, these data reveal a direct role for c-MYC in the transcriptional regulation of RRN3 and POLR1B and given the correlating expression and presence of E-boxes in regulatory regions of other Pol I regulon members, they too are most likely directly regulated by MYC.

c-MYC enables recruitment of the Pol I apparatus to the rDNA repeat but does not alter the relative proportion of active rRNA genes in terminally differentiated neutrophils

We have demonstrated that MYC can promote changes in active gene number during TGD; in addition, we have

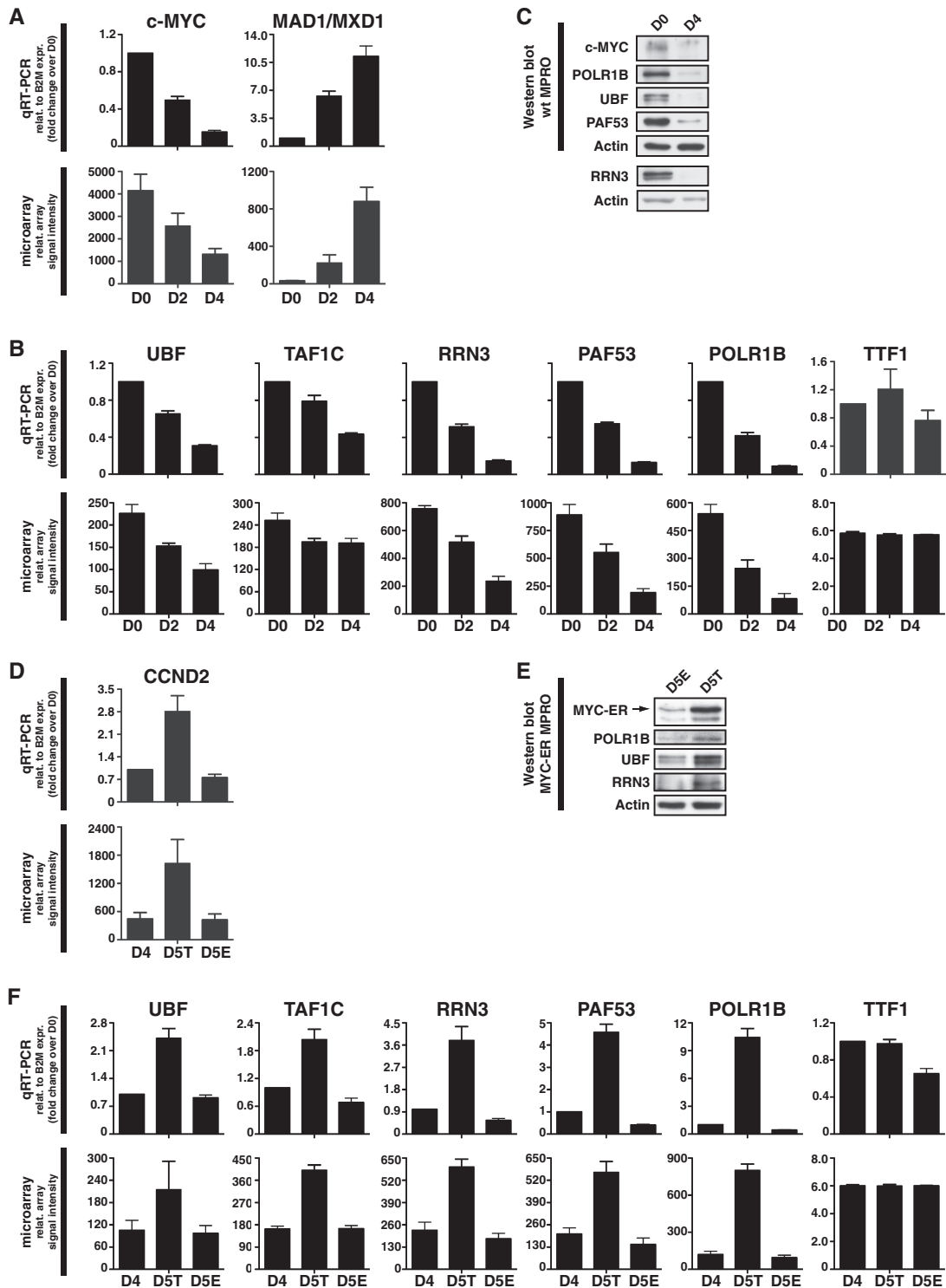


Figure 3. Validation of the gene expression array data defining a MYC regulated Pol I regulon. (A) Wt MPROs were harvested for RNA extraction prior to differentiation (D0) and after 2 (D2) and 4 (D4) days of differentiation. qRT-PCR (top panels) and gene expression analysis (signal intensity, bottom panels) were performed to assay expression of MYC and MAD1. (B) qRT-PCR (top) and gene expression analysis (bottom) were performed as described above to assay expression of Pol I regulon members: UBF, TAF1C, RRN3, PAF53 and POLR1B. TTF-1 was also assayed as a non-regulon member. (C) Protein lysates made from wt MPRO cells treated as described in (A) (D0 and D4 only) were analysed by Western blotting for expression of MYC and Pol I regulon members as compared to the Actin loading control. (D) Differentiated MYC-ER MPROs (D4) were treated with EtOH vehicle or 4-OHT for 24 h (D5E and D5T, respectively) before harvesting for RNA extraction. qRT-PCR (top) and gene expression analysis (bottom) were performed to assay expression of CCND2. (E) Protein lysates made from MYC-ER cells treated as described in (D) (D5E and D5T only) were analysed by Western blotting for expression of MYC-ER, POLR1B, UBF and RRN3 compared to Actin. (F) MYC-ER cells were treated as described in (D) and qRT-PCR (top) and gene expression analysis (bottom) were performed to assay expression of Pol I regulon members and TTF-1. All qRT-PCR data was normalized to B2M expression. Results for all graphed data are the mean \pm SEM from four independent experiments.

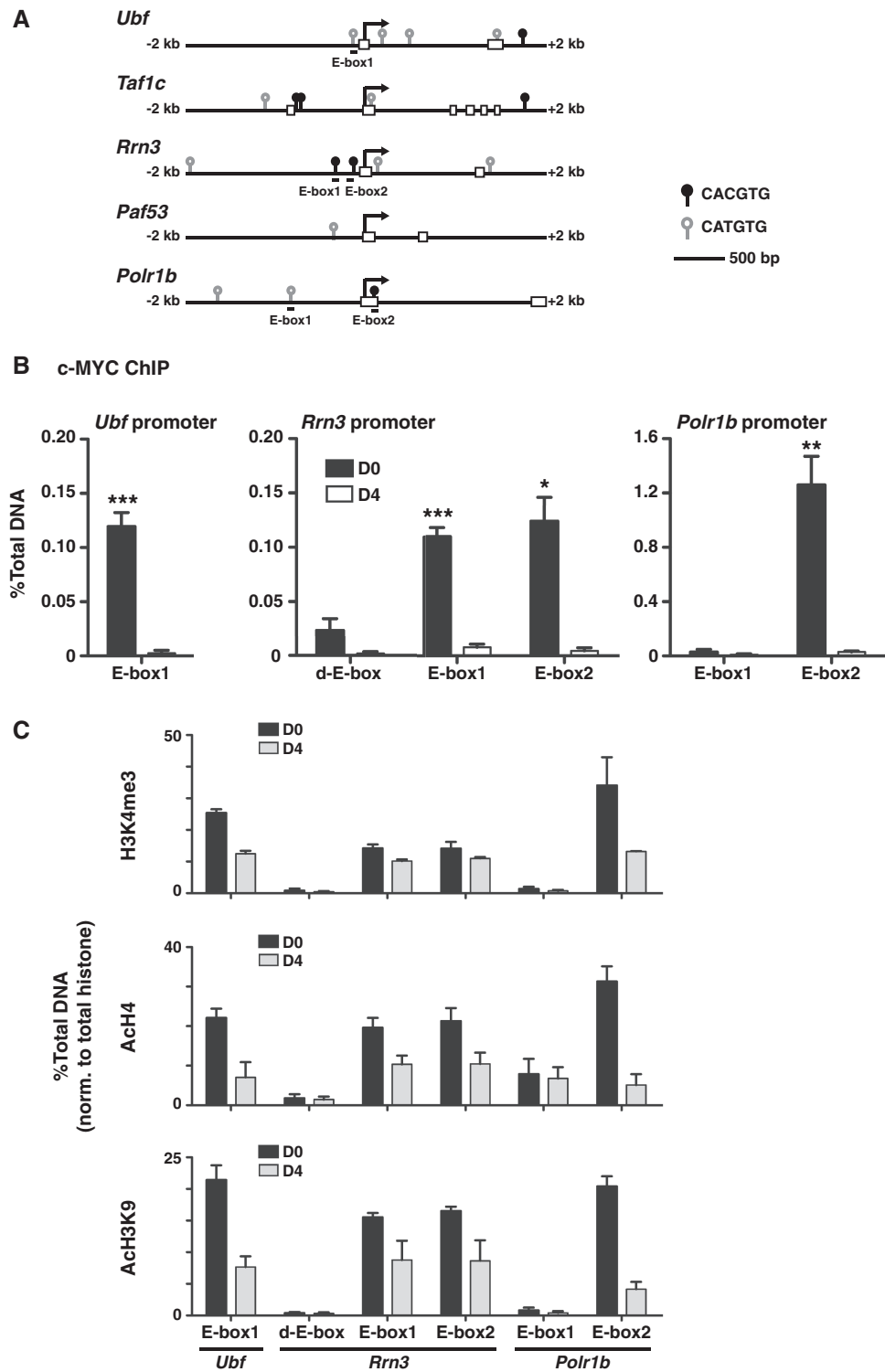


Figure 4. Pol I regulon members are direct c-MYC transcriptional targets. (A) Schematics of promoter regions and nearby exons of select Pol I regulon genes. 2 kb up- and downstream of the TSS (arrows) are represented and canonical (solid black circles) and non-canonical (open grey circles) E-boxes are indicated. Amplicons used for qChIP analysis in (B) and (C) are identified with a bar below the corresponding sequence, except the *Rrn3* distal E-box (d-E-box), located 4 kb upstream of the TSS. (B) qChIP analysis of MYC binding at E-boxes at target Pol I regulon genes *Ubf*, *Rrn3* and *Polr1b*. Wt MPROs were differentiated and qChIP analysis was performed as described in Figure 1D using the anti-c-MYC antibody and control rabbit IgG. Results are the mean \pm SEM from a minimum of five independent experiments for all amplicons except *Polr1b* E-box1 ($n = 3$). * $P < 0.01$, ** $P = 0.001$ and *** $P < 5.0 \times 10^{-5}$ compared to D4. (C) qChIP analysis in D0 and D4 wt MPROs using anti-trimethyl H3K4, anti-acetylated H4 and anti-acetylated H3K9 antibodies versus control antisera at E-boxes analyzed in (B) and carried out as described in Figure 1D with the total percentage ChIP values normalized to the mean total percentage for either histone H3 (H3K4me3 and H3K9ac) or H4 (H4ac) at respective amplicons (Supplementary Figure S5A). Results are the mean \pm SD from a minimum of two independent experiments.

provided evidence that MYC is required for the maintenance of expression of the Pol I regulon suggesting that as MYC levels decline during TGD, the amount of available Pol I complexes becomes limiting. Activation of MYC-ER in differentiated MPRO cells leads to a greater than 2-fold increase in 45S expression (Figure 2D) and a concomitant induction of core Pol I transcription factors (Figure 3E and F). Thus, these cells provide an ideal MYC gain-of-function setting to investigate both modulation of active rRNA gene number and levels of Pol I complex associated with the rDNA repeat as potential mechanisms of MYC-mediated regulation of 45S transcription in the MPRO system. Following differentiation, D4 MPROs were exposed to 4-OHT for 24 h and subsequent psoralen analysis showed that while these cells displayed the typical shift from predominantly active rRNA genes at D0 to inactive at D4, once differentiated the majority of rRNA genes remained inactive despite activation of MYC and induction of MYC targets including UBF, a regulator of active rRNA gene number (Figures 3E, F and 5A). We then carried out a qChIP analysis of UBF and found that consistent with maintenance of a low active to inactive gene ratio there was no significant change in the amount of UBF associated with the rDNA repeat regardless of UBF protein induction by MYC-ER (Figures 3E and 5B, left).

An alternative explanation for MYC stimulation of rRNA transcription in differentiated MPROs is increased recruitment of Pol I complexes to the rRNA gene promoter via the observed c-MYC-ER induced expression

of the Pol I regulon (Figures 2F, 3E and F). We performed qChIP analysis of Pol I in D5T MPROs and found that the same rDNA regions displaying significant binding by Pol I in proliferating MPROs, e.g. the ETS1 and 2 sites, likewise displayed a 2-fold or more increase in Pol I occupancy compared with control D5E cells (Figure 5B, right). Thus, while MYC induced 45S expression coincides with an increased association of the core POLR1B subunit at the rDNA repeat, the lack of enrichment of UBF or changes in active rRNA gene number suggests that once cells have terminally differentiated MYC mediates activation of rDNA transcription independent of alterations in the number of active rRNA genes (Figure 5B).

DISCUSSION

The MYC proto-oncogene transcriptionally regulates a growing list of genes representing most of the processes that drive cellular growth (49,50). Central to this function is the ability of MYC to control transcription of the ribosomal genes, one of the most fundamental rate-limiting steps in growth control (3). What is less clear is how MYC achieves growth control over rDNA transcription with numerous mechanism being implicated, including indirect control through MYC dependent transcriptional regulation of the Pol I factors such as UBF, direct mechanisms such as protein-protein interactions between MYC and Pol I initiation factors and direct binding of MYC to the rDNA repeat and finally, regulation of rRNA precursor processing (6-9,23). In this study, we

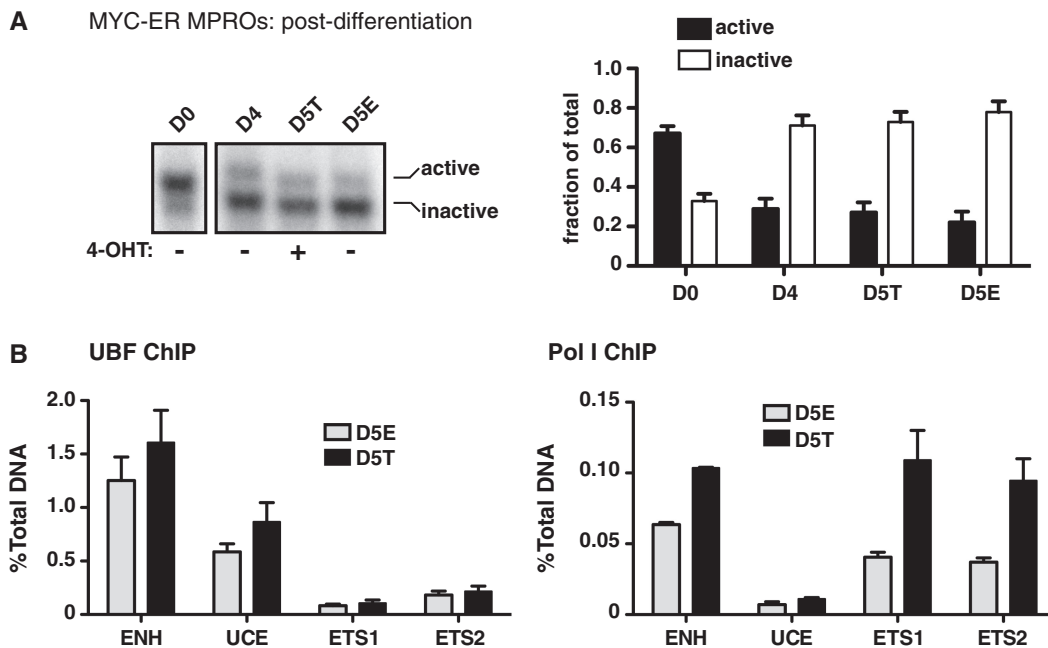


Figure 5. MYC activation is sufficient to recruit Pol I but not to change active rRNA gene ratio in terminally differentiated neutrophils. (A) Proliferating MYC-ER MPROs (D0) were induced to differentiate for 4 days (D4) prior to treatment with either EtOH vehicle or 4-OHT for 24 h (D5E and D5T, respectively) then harvested for psoralen crosslinking analysis as described in Figure 1B. A representative Southern blot of rRNA genes (left) and quantitated active and inactive gene fractions (right) are shown. Results are the mean \pm SEM from three independent experiments. (B) MYC-ER MPROs were treated and harvested as in (A) and qChIP analysis for enrichment of select rDNA amplicons from Figure 1C obtained with anti-UBF (left) and anti-Pol I (right) was performed on D5E and D5T samples as described in Figure 1D (UBF ChIP, $n = 3$; Pol I ChIP, $n = 2$).

have examined the ability of MYC to control rDNA transcription in a physiologically relevant model of granulocyte differentiation where the programmed downregulation of MYC expression as cells differentiate is associated with a marked repression of rDNA transcription.

We demonstrate that MYC controls rDNA transcription during differentiation at two distinct levels. First, through direct regulation of the chromatin remodeling factor UBF, MYC regulates the number of rRNA gene copies that have an open chromatin structure and are thus transcriptionally active; second, we show that MYC modulates the loading of Pol I onto the active pool of rRNA genes by transcriptionally controlling the availability of core components of the Pol I initiation complex. Thus, MYC functions to balance the number of active rRNA genes with levels of the Pol I transcription apparatus to ensure that loading of Pol I onto the rRNA genes remains optimal and matches ongoing demand for rDNA transcription. Thus, in the highly proliferative granulocyte precursors, where demand for rDNA transcription is high, elevated MYC levels ensure the number of transcriptionally active ribosomal genes is maximal as is the availability of active Pol I. Conversely in terminally differentiated neutrophils where the need for rRNA is reduced, negligible MYC expression ensures the number of transcriptionally active rRNA genes is low and the availability of active Pol I is limiting. Lastly, we demonstrate that induction of MYC is sufficient to reactivate expression of the Pol I regulon and rDNA transcription in terminally differentiated cells even though its ability to increase the number of active rRNA genes is lost. The uncoupling of the ability of MYC to coordinate active rRNA gene number and the level of Pol I suggests that terminally differentiated cells have evolved a chromatin status that 'clamps' Pol I transcription capacity at a reduced range, possibly to prevent unwanted re-activation of ribosome biogenesis which may be counter-productive or even detrimental to non-dividing cells.

Myc regulation of r-chromatin via UBF

At any one time, over 50% of the 200 copies of rRNA genes are transcriptionally silent due to methylation of specific CpGs at promoter proximal regions (4,5). The remaining 50% are unmethylated and transcriptionally active as long as they are bound by UBF that functions to maintain an open chromatin structure of the unmethylated rRNA genes, most likely by displacing linker H1 (51). Here we demonstrate that not only did activation of MYC via a *MYC-ER* transgene prior to differentiation prevent the decrease in active gene number but also activation of MYC during differentiation reversed the decrease in number of active genes. We were unable to effectively deplete UBF protein for the duration necessary to determine the extent of its requirement by MYC for modulation of active rRNA gene number due to a strong selection against loss of UBF in the highly proliferative MPROs. Nonetheless, this data indicates that MYC regulates the proportion of active rRNA genes at least in part through its ability to

transcriptionally control UBF levels and thus UBF loading on the unmethylated rRNA genes and consequently, in addition to its previously described roles in regulation of rDNA transcription rate per gene, MYC also controls chromatin remodeling of unmethylated rRNA genes.

Myc regulation of a Pol I regulon

In other systems silencing of unmethylated rRNA genes by depletion of UBF leads to a compensatory increase in loading of Pol I on the remaining active genes, thus maintaining rDNA transcription rates (12,24). In contrast, during TGD, even accounting for the reduced number of active rRNA genes, Pol I loading per gene in the differentiated cells was dramatically lower than in undifferentiated cells, suggesting that additional processes are required to block increased Pol I loading onto the remaining active rRNA genes. Our data demonstrate that the most likely mechanism to account for this is that the availability of the Pol I transcription apparatus becomes limiting for efficient rDNA transcription. Expression array analysis demonstrates that during differentiation a suite of genes important for Pol I transcription are significantly downregulated. Furthermore our data strongly implicate MYC in the regulation of this Pol I regulon as reactivation of MYC in differentiated cells, where MYC levels are normally low, led to induction of these genes. In fact when Pol I-, II- and III- specific gene profiles were examined the most significant changes in gene expression clustered with the Pol I regulon. Overall, induction of MYC-ER in the differentiated MPROs coincided with a 2-fold or greater increase in expression of 1897 genes or about 8% of genes represented on the gene expression array chip. Over 79% of known Pol I-specific as compared with only 14% of Pol II- specific factors were upregulated in this setting. Furthermore, a selection of Pol I regulon genes analyzed had one or more E-boxes in their promoter proximal regions and for the two of these tested, MYC directly bound the promoters by qChIP analysis. Thus, we conclude that MYC transcriptionally regulates the Pol I regulon during TGD to control the level of Pol I factors available for efficient transcription. Interestingly, in the absence of E-boxes or direct dMyc interaction at rRNA genes, an indirect role for MYC regulation of Pol I factors was observed in *Drosophila* (52). This reinforces that such modulation is less likely to be critical for acute regulation of rDNA transcription in response to flux in growth factor signaling, however during development and differentiation or when new steady state levels of rDNA transcription need to be established, controlling the level of available Pol I factors provides an attractive mechanism to achieve sustainable regulation of Pol I transcription.

Does MYC regulate Pol I by other mechanisms in differentiating granulocytes?

While the availability of Pol I factors are likely to be important for Pol I loading, clearly additional regulatory process such as histone modifications are also required to facilitate efficient Pol I transcription initiation. In this

regard it is of interest to note that others have reported data that MYC is able to regulate rDNA transcription by interaction with the TBP containing SL-1 factor, direct rDNA binding and more recently, via facilitation of rDNA gene looping (7–9). In the MPRO differentiation model absolute levels of endogenous MYC protein associated with the rDNA repeat were considerably low as compared with levels of MYC bound to the Pol II regulated MYC target genes described in this study (Figure 4B and compared with *UBF* in Supplementary Figure S1A). While the consequence of this comparatively low but statistically significant level of MYC binding at the rDNA repeat is unclear, the highest occupancy of MYC was observed within the ETS region proximal to the TSS and also in the transcription termination region, regions similarly located to the amplicons showing the highest c-MYC occupancy at the human rDNA repeat (8). It is possible that the functional threshold for MYC binding at the rDNA repeat is lower than that at Pol II target genes where MYC transcriptional activity can be regarded as relatively inefficient (2). In addition, this observed low-level association of endogenous MYC at the rDNA repeat may mediate functions other than acute transcriptional regulation, such as effecting changes on the chromatin topography associated with maintenance of the undifferentiated state or facilitating assembly of the high levels of Pol I components required for transcription. This last point includes the ability of MYC to interact with SL-1 and assist its loading onto the rDNA repeat whereby MYC itself, even at low occupancy, may be a component of the Pol I machinery that becomes limiting during TGD (8). Finally, there is emerging evidence that demonstrates a role for MYC, particularly in the context of maintaining cellular pluripotency (e.g. ES cells), in the more generalized epigenetic regulation of chromatin (53–55).

MYC coordinates active gene number with availability of the Pol I apparatus to ensure long-term regulation of Pol I transcription

An intriguing question that arises from our studies is why differentiating cells have evolved a mechanism to silence rRNA genes if it is not permanently sufficient to downregulate Pol I transcription. However, we propose that the coordinated modulation of both active rRNA gene number and levels of available Pol I apparatus are essential to allow for efficient downregulation of rDNA transcription during differentiation. Firstly, a reduction in number of active genes without concomitant reduction in the level of Pol I could result in the hyperactivation of remaining genes, with the net effect being sustained high rDNA transcription levels not compatible with differentiation. Conversely, a reduction in only the levels of Pol I components without concomitant silencing of active rRNA genes would lead to a large proportion of ‘open’ rRNA genes devoid of Pol I; recent studies by Gagnon-Kugler *et al.* (56) suggest that this could lead to their transcription by Pol II, an event shown to be cytotoxic to the cell. By coordinating these two processes, the

lower steady state levels of rRNA synthesis reflective of differentiated granulocytes can be achieved while preventing incursion of Pol II into the Pol I locus or other potential detrimental effects associated with open r-chromatin that is not undergoing active Pol I transcription. Interestingly, coupling of the levels of active rRNA genes and Pol I activity is not obligate as in the terminally differentiated granulocytes, forced reactivation of MYC no longer increases the number of active rRNA genes although it does increase Pol I regulon levels, Pol I loading onto rDNA repeats and rDNA transcription, although to significantly lower levels than those found in the proliferating cells. This ‘lock’ on the inactive chromatin state may provide cells with an assurance against erroneous hyperactivation of rDNA transcription that is incompatible with the differentiated cell program. We recently reported that methylation of a crucial regulatory CpG di-nucleotide at –133 in the rRNA promoter implicated in silencing of murine rRNA genes (38) and shown to reduce UBF binding to the rRNA gene promoter does not change during TGD in MPRO cells (12). Thus, it does not appear that the inability of MYC to increase the number of active repeats in differentiated MPROs is regulated by methylation dependent silencing. We have previously described this form of silencing, termed pseudosilencing, in which UBF is unable to be loaded onto rDNA independent of CpG methylation (12). It may be the case that the repeat acquires other chromatin marks, potentially acquired in the absence of c-MYC’s epigenetic programming influences that prevent the reversal of their inactive status.

In conclusion, we propose that MYC functions during differentiation to coordinate the pool of active rRNA genes with levels of Pol I factors to ensure the tight regulation of rDNA transcription and ultimately ribosome biogenesis to match cellular needs at any given stage. It is interesting to speculate that MYC driven cancers associated with deregulation of MYC might be associated with proportionate increases in the number of active rRNA genes and levels of Pol I factors which may account for the hyperactivation of Pol I transcription associated with these malignancies. Intriguingly, our preliminary data demonstrate that MYC driven B-cell lymphoma exhibit 5-fold increases in active rRNA gene number that correlate with increased UBF expression and UBF loading on active repeats (M. J. Bywater, manuscript in preparation). We are currently investigating whether MYC regulation of UBF and this observed increase in active gene number contribute to the malignant process.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

- Oskarsson, T. and Trumpp, A. (2005) The Myc trilogy: lord of RNA polymerases. *Nat. Cell Biol.*, **7**, 215–217.
- Gomez-Roman, N., Felton-Edkins, Z.A., Kenneth, N.S., Goodfellow, S.J., Athineos, D., Zhang, J., Ramsbottom, B.A., Innes, F., Kantidakis, T., Kerr, E.R. *et al.* (2006) Activation by c-Myc of transcription by RNA polymerases I, II and III. *Biochem. Soc. Symp.*, **73**, 141–154.
- Jorgensen, P. and Tyers, M. (2004) How cells coordinate growth and division. *Curr. Biol.*, **14**, R1014–R1027.
- Grummt, I. and Pikaard, C.S. (2003) Epigenetic silencing of RNA polymerase I transcription. *Nat. Rev. Mol. Cell Biol.*, **4**, 641–649.
- McStay, B. and Grummt, I. (2008) The epigenetics of rRNA genes: from molecular to chromosome biology. *Annu. Rev. Cell Dev. Biol.*, **24**, 131–157.
- Poortinga, G., Hannan, K.M., Snelling, H., Walkley, C.R., Jenkins, A., Sharkey, K., Wall, M., Brandenburger, Y., Palatsides, M., Pearson, R.B. *et al.* (2004) MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. *EMBO J.*, **23**, 3325–3335.
- Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fathyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I. *et al.* (2005) c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat. Cell Biol.*, **7**, 303–310.
- Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N. and White, R.J. (2005) c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat. Cell Biol.*, **7**, 311–318.
- Shiue, C.N., Berkson, R.G. and Wright, A.P. (2009) c-Myc induces changes in higher order rDNA structure on stimulation of quiescent cells. *Oncogene*, **28**, 1833–1842.
- Chen, D., Belmont, A.S. and Huang, S. (2004) Upstream binding factor association induces large-scale chromatin decondensation. *Proc. Natl Acad. Sci. USA*, **101**, 15106–15111.
- Mais, C., Wright, J.E., Prieto, J.L., Raggett, S.L. and McStay, B. (2005) UBF-binding site arrays form pseudo-NORs and sequester the RNA polymerase I transcription machinery. *Genes Dev.*, **19**, 50–64.
- Sanij, E., Poortinga, G., Sharkey, K., Hung, S., Holloway, T.P., Quin, J., Robb, E., Wong, L.H., Thomas, W.G., Stefanovsky, V. *et al.* (2008) UBF levels determine the number of active ribosomal RNA genes in mammals. *J. Cell Biol.*, **183**, 1259–1274.
- O'Sullivan, A.C., Sullivan, G.J. and McStay, B. (2002) UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol. Cell Biol.*, **22**, 657–668.
- Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A. and Amati, B. (2003) Genomic targets of the human c-Myc protein. *Genes Dev.*, **17**, 1115–1129.
- Li, Z., Van Calcar, S., Qu, C., Cavenee, W.K., Zhang, M.Q. and Ren, B. (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc. Natl Acad. Sci. USA*, **100**, 8164–8169.
- Patel, J.H., Loboda, A.P., Showe, M.K., Showe, L.C. and McMahon, S.B. (2004) Analysis of genomic targets reveals complex functions of MYC. *Nat. Rev. Cancer*, **4**, 562–568.
- Zeller, K.I., Zhao, X., Lee, C.W., Chiu, K.P., Yao, F., Yustein, J.T., Ooi, H.S., Orlov, Y.L., Shahab, A., Yong, H.C. *et al.* (2006) Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proc. Natl Acad. Sci. USA*, **103**, 17834–17839.
- Hoffman, B., Amanullah, A., Shafarenko, M. and Liebermann, D.A. (2002) The proto-oncogene c-myc in hematopoietic development and leukemogenesis. *Oncogene*, **21**, 3414–3421.
- Wall, M., Poortinga, G., Hannan, K.M., Pearson, R.B., Hannan, R.D. and McArthur, G.A. (2008) Translational control of c-MYC by rapamycin promotes terminal myeloid differentiation. *Blood*, **112**, 2305–2317.
- Nguyen, H.Q., Selvakumaran, M., Liebermann, D.A. and Hoffman, B. (1995) Blocking c-Myc and Max expression inhibits proliferation and induces differentiation of normal and leukemic myeloid cells. *Oncogene*, **11**, 2439–2444.
- Amanullah, A., Liebermann, D.A. and Hoffman, B. (2000) p53-independent apoptosis associated with c-Myc-mediated block in myeloid cell differentiation. *Oncogene*, **19**, 2967–2977.
- McArthur, G.A., Foley, K.P., Fero, M.L., Walkley, C.R., Deans, A.J., Roberts, J.M. and Eisenman, R.N. (2002) MAD1 and p27(KIP1) cooperate to promote terminal differentiation of granulocytes and to inhibit Myc expression and cyclin E-CDK2 activity. *Mol. Cell Biol.*, **22**, 3014–3023.
- Schlosser, I., Holz, M., Murnseer, M., Burtcher, H., Weidle, U.H. and Eick, D. (2003) A role for c-Myc in the regulation of ribosomal RNA processing. *Nucleic Acids Res.*, **31**, 6148–6156.
- French, S.L., Osheim, Y.N., Cioci, F., Nomura, M. and Beyer, A.L. (2003) In exponentially growing *Saccharomyces cerevisiae* cells, rRNA synthesis is determined by the summed RNA polymerase I loading rate rather than by the number of active genes. *Mol. Cell Biol.*, **23**, 1558–1568.
- Ide, S., Miyazaki, T., Maki, H. and Kobayashi, T. (2010) Abundance of ribosomal RNA gene copies maintains genome integrity. *Science*, **327**, 693–696.
- Tsai, S. and Collins, S.J. (1993) A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocyte stage. *Proc. Natl Acad. Sci. USA*, **90**, 7153–7157.
- Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G. and Evan, G.I. (1995) A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res.*, **23**, 1686–1690.
- Hannan, K.M., Brandenburger, Y., Jenkins, A., Sharkey, K., Cavanaugh, A., Rothblum, L., Moss, T., Poortinga, G., McArthur, G.A., Pearson, R.B. *et al.* (2003) mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol. Cell Biol.*, **23**, 8862–8877.
- Hannan, K.M., Kennedy, B.K., Cavanaugh, A.H., Hannan, R.D., Hirschler-Laszkiewicz, I., Jefferson, L.S. and Rothblum, L.I. (2000) RNA polymerase I transcription in confluent cells: Rb downregulates rDNA transcription during confluence-induced cell cycle arrest. *Oncogene*, **19**, 3487–3497.
- Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.*, **5**, R80.
- Bolstad, B.M., Collin, F., Brettschneider, J., Simpson, K., Cope, L., Irizarry, R.A. and Speed, T.P. (2005) Quality Assessment of Affymetrix GeneChip Data. In Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A. and Dudoit, S. (eds), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer, New York, pp. 33–47.
- Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B. and Speed, T.P. (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.*, **31**, e15.
- Gautier, L., Cope, L., Bolstad, B.M. and Irizarry, R.A. (2004) affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*, **20**, 307–315.
- Smyth, G.K. (2005) Limma: linear models for microarray data. In Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A. and Dudoit, S. (eds), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer, New York.
- Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. Ser. B*, **57**, 289–300.

36. Conconi,A., Widmer,R.M., Koller,T. and Sogo,J.M. (1989) Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. *Cell*, **57**, 753–761.
37. Dammann,R., Lucchini,R., Koller,T. and Sogo,J.M. (1993) Chromatin structures and transcription of rDNA in yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 2331–2338.
38. Santoro,R. and Grummt,I. (2001) Molecular mechanisms mediating methylation-dependent silencing of ribosomal gene transcription. *Mol. Cell*, **8**, 719–725.
39. Lawrence,R.J., Earley,K., Pontes,O., Silva,M., Chen,Z.J., Neves,N., Viegas,W. and Pikaard,C.S. (2004) A concerted DNA methylation/histone methylation switch regulates rRNA gene dosage control and nucleolar dominance. *Mol. Cell*, **13**, 599–609.
40. Eilers,M., Schirm,S. and Bishop,J.M. (1991) The MYC protein activates transcription of the alpha-prothymosin gene. *EMBO J.*, **10**, 133–141.
41. Hannan,K.M., Hannan,R.D. and Rothblum,L.I. (1998) Transcription by RNA polymerase I. *Front. Biosci.*, **3**, d376–d398.
42. Moss,T., Langlois,F., Gagnon-Kugler,T. and Stefanovsky,V. (2007) A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell Mol. Life Sci.*, **64**, 29–49.
43. Nemeth,A., Strohner,R., Grummt,I. and Langst,G. (2004) The chromatin remodeling complex NoRC and TTF-I cooperate in the regulation of the mammalian rRNA genes in vivo. *Nucleic Acids Res.*, **32**, 4091–4099.
44. Nemeth,A., Guibert,S., Tiwari,V.K., Ohlsson,R. and Langst,G. (2008) Epigenetic regulation of TTF-I-mediated promoter-terminator interactions of rRNA genes. *EMBO J.*, **27**, 1255–1265.
45. Guccione,E., Martinato,F., Finocchiaro,G., Luzzi,L., Tizzoni,L., Dall’Olio,V., Zardo,G., Nervi,C., Bernard,L. and Amati,B. (2006) Myc-binding-site recognition in the human genome is determined by chromatin context. *Nat. Cell Biol.*, **8**, 764–770.
46. Martinato,F., Cesaroni,M., Amati,B. and Guccione,E. (2008) Analysis of Myc-induced histone modifications on target chromatin. *PLoS One*, **3**, e3650.
47. Eilers,M. and Eisenman,R.N. (2008) Myc’s broad reach. *Genes Dev.*, **22**, 2755–2766.
48. Frank,S.R., Parisi,T., Taubert,S., Fernandez,P., Fuchs,M., Chan,H.M., Livingston,D.M. and Amati,B. (2003) MYC recruits the TIP60 histone acetyltransferase complex to chromatin. *EMBO Rep.*, **4**, 575–580.
49. Ruggero,D. (2009) The role of Myc-induced protein synthesis in cancer. *Cancer Res.*, **69**, 8839–8843.
50. van Riggelen,J., Yetil,A. and Felsner,D.W. (2010) MYC as a regulator of ribosome biogenesis and protein synthesis. *Nat. Rev. Cancer*, **10**, 301–309.
51. Sanij,E. and Hannan,R.D. (2009) The role of UBF in regulating the structure and dynamics of transcriptionally active rDNA chromatin. *Epigenetics*, **4**, 374–382.
52. Grewal,S.S., Li,L., Orian,A., Eisenman,R.N. and Edgar,B.A. (2005) Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development. *Nat. Cell Biol.*, **7**, 295–302.
53. Knoepfler,P.S. (2007) Myc goes global: new tricks for an old oncogene. *Cancer Res.*, **67**, 5061–5063.
54. Knoepfler,P.S., Zhang,X.Y., Cheng,P.F., Gafken,P.R., McMahon,S.B. and Eisenman,R.N. (2006) Myc influences global chromatin structure. *EMBO J.*, **25**, 2723–2734.
55. Lin,C.H., Lin,C., Tanaka,H., Fero,M.L. and Eisenman,R.N. (2009) Gene regulation and epigenetic remodeling in murine embryonic stem cells by c-Myc. *PLoS One*, **4**, e7839.
56. Gagnon-Kugler,T., Langlois,F., Stefanovsky,V., Lessard,F. and Moss,T. (2009) Loss of human ribosomal gene CpG methylation enhances cryptic RNA polymerase II transcription and disrupts ribosomal RNA processing. *Mol. Cell*, **35**, 414–425.