c-Myc induced changes in higher order rDNA structure accompany growth factor stimulation of quiescent cells

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Human c-Myc is believed to be a high level coordinator of protein synthesis capacity and cell growth rate, capable of activating transcription by all three nuclear RNA Polymerases. Direct activation of rDNA transcription by c-Myc is functionally conserved in rat cells, despite high divergence in non-coding rDNA sequences, suggesting that this coordinating role is likely to be a general within mammals. Upon refeeding of starved cells, c-Myc activity enhances the efficiency of RNA Polymerase I and SL1/TIF-1B recruitment to the rDNA and rapidly induces higher order gene loop structures in rDNA chromatin that juxtapose upstream and downstream rDNA sequences. Furthermore c-Myc induced gene-loop formation in rDNA genes occurs independently of rDNA transcription, implying that it may be an early step in the re-programming of quiescent cells as they enter the growth cycle.

The c-Myc transcription factor is deregulated in a wide range of human cancers[1]. c-Myc regulates genes involved in a range of processes, notably cell growth and proliferation[2]. c-Myc is associated with many locations throughout the genome, but it has been hard to experimentally characterise c-Myc as potent regulator of individual target genes[3]. Some recent evidence suggests that c-Myc may contribute to gene regulation at a more global level[4]. Recent evidence suggests that human c-Myc stimulates cell growth, in part via its ability to directly activate transcription of genes encoding both the protein and RNA components of the protein synthesis machinery, which are transcribed by RNA Polymerases II, and I and III respectively[3]. Protein synthesis capacity is rate limiting for cell growth and the coordinated production of ribosomal components in stoichiometric amounts is important for successful ribosome biogenesis[5].

It has been shown previously that c-Myc binds to sequences upstream and downstream of the rRNA coding regions in the human rDNA that contain putative c-Myc binding sites (E-boxes)[6]. To better understand the role of c-Myc in mammalian

cells we compared human, mouse and rat rDNA sequences. As expected the regions encoding the mature 18S, 5.8S and 28S rRNAs are highly conserved. However, the non-transcribed regions and non-coding transcribed regions are much more variable (see Supplementary Fig. S1 online). In particular, the sequences upstream and downstream of the rRNA coding regions are most diverged in the rat (c. 20% sequence conservation compared to human). The number of E-boxes in the rat sequence (Fig. 1a) is lower than in either the human or mouse sequences and the position of E-boxes in the sequences is not conserved. c-Myc induces rDNA transcription in rat cells[7] but it is not known whether this involves direct interaction with the rDNA as in human cells. In fruit flies functional E-boxes are not found in the rDNA and dMyc induces rDNA transcription indirectly[8].

To determine whether c-Myc associates with rat rDNA sequences as with the equivalent but divergent human upstream and downstream regions, we performed chromatin immunoprecipitation experiments in Rat1 fibroblasts. Fig. 1b shows that c-Myc associated with both upstream and downstream regions in serum stimulated *c-myc*+/+ rat fibroblasts (TGR1). No significant association is seen without serum stimulation or in *c-myc*-/- cells (HO1519). Consistently, c-Myc is required for efficient serum-dependent induction of rDNA transcription in Rat1 cells (Fig. 1c). Specific activation of a stably expressed c-Myc/ER fusion protein in serum-starved *c-myc*-/- cells using the Estrogen Receptor ligand, 4-hydroxytamoxifen (4-HT), is sufficient to induce rDNA transcription within 2h (Fig. 1d). This rapid, growth factor independent response strongly suggests direct activation of rDNA transcription by rDNA-associated c-Myc. Myc-induced rDNA transcription was still seen in cells pre-treated with α-amanitin to inhibit transcription by RNA Polymerase II (see

Supplementary Fig. S2 online) as previously seen in human cells[6,7]. We conclude that c-Myc is a direct regulator of rat rDNA transcription and that this role is likely to be a functionally conserved feature within mammals despite the high divergence of non-coding rDNA sequences and the E-boxes they contain.

To further characterise the mechanism by which c-Myc activates rDNA transcription we used the Rat1 fibroblast-derived cell lines to investigate the role of c-Myc in recruitment of transcription complex components to the rDNA. Fig 2a shows that c-Myc is required for efficient recruitment of polymerase I (RPA 194) and the TATA binding protein (TBP) to sequences upstream of the rRNA coding regions upon serum activation. TBP is a component of the RNA Polymerase I specific factor, SL1/TIF-1B[9]. Recruitment to the promoter and upstream enhancer is also seen in *c-myc-/-* cells but at a lower level than in the presence of c-Myc. Much lower levels of RNA Polymerase I and TBP were found downstream of the transcription termination site (R+13) as expected. Surprisingly, we observed strong association of both RNA Polymerase I and TBP further downstream of the rRNA transcription termination site (R+17) in serum-stimulated, c-Myc expressing cells. Furthermore, Fig 2b shows that specific c-Myc activation by 4-HT in serum-starved cells expressing Myc/ER induces a similar pattern of RNA Polymerase I and TBP binding to the rDNA. The c-Myc dependent association of RNA Polymerase I and TBP to non-transcribed sequences downstream of the rRNA transcription unit has not been observed previously and we therefore decided to investigate this aspect further.

Given the well-characterised role of TBP as a factor involved in transcriptional initiation[10], we speculated that the downstream signal could result from physical

juxtaposition of upstream and downstream rDNA regions during rDNA transcription rather than from direct association of TBP with the downstream region. Juxtaposition of upstream and downstream regions causes gene loops in several RNA Polymerase II transcribed genes in yeast[11], and is thought to facilitate transcriptional reinitiation by passing newly terminated RNA polymerases back to the promoter[12].

To determine whether gene loops are formed in mammalian rDNA we used the chromosome conformation capture (3C) technique[13], which identifies restriction enzyme cleaved ends of DNA that can be efficiently religated to each other by virtue of their close proximity in higher order chromatin structures. Chromatin preparations from growing cells were cleaved with *HindIII*, or *BamHI* and religated under dilute conditions under which only closely juxtaposed cohesive ends can be joined. Ends that are juxtaposed were then identified by PCR using combinations of primers pairs that lie close to the restriction enzyme cleavage sites. The location of cleavage and primer sites on the rat rDNA is shown in Fig. 3a. Fig. 3b shows fragments amplified by different primer combinations after cleavage with the different enzymes and religation. For many primer pairs no specific fragment is amplified, as expected for DNA regions that are not juxtaposed by a higher order chromatin structure. However, DNA ends associated with fragments containing upstream and downstream regions can be religated after cleavage with each of the enzymes leading to the amplification of the ligated products (primer sets 1+8, 2+5, 2+6, 2+7). Importantly, all the positive signals are dependent on addition of DNA ligase to the ligation reactions and are lost if the conservation of chromatin structures during extract preparation is compromised by the omission of the cross-linking step. We conclude that the rDNA in growing

cells forms a higher order chromatin structure in which the upstream and downstream regions of rDNA genes are juxtaposed.

Nucleolar morphology depends on rDNA gene structure and transcription[14-16]. Given the large number of tandemly repeated rDNA genes, loops in the rDNA could drive the changes in nucleolar morphology accompanying growth factor stimulation of rDNA transcription and cell growth. We used the 3C technique to determine whether rDNA gene looping is enhanced upon serum stimulation of starved cells and whether c-Myc is involved. Fig. 4a shows that juxtaposition of upstream and downstream rDNA sequences is induced in response to serum treatment. c-Myc is required for efficient gene loop formation because gene loop formation was not detectable in *c-myc*$^{-/-}$ cells. We wondered whether activation of c-Myc in starved cells is sufficient to induce gene looping. Furthermore, we wondered whether c-Myc would induce loop formation directly or as a consequence of enhanced rDNA transcription. Fig. 4b shows that 4-HT treatment of starved c-Myc/ER containing cells also causes rDNA gene looping and therefore activation of c-Myc is sufficient for gene loop induction. Importantly, c-Myc induced rDNA gene looping is seen in cells that have been pre-treated with Actinomycin D to inhibit rDNA transcription. Therefore, c-Myc induced gene looping in the rDNA is not dependent on induced levels of rDNA transcription, and thus may be a cause of induced transcription rather than its consequence. Fig. 4c shows that the Actinomycin D treatment effectively inhibits rDNA transcription under the conditions used. A causative role of gene looping in rDNA activation would require rapid gene loop formation since c-Myc induced rDNA transcription is observed 2 hours after c-Myc activation (see Fig. 1d). Fig 4d shows that rDNA gene loops are fully induced by 30 minutes after c-Myc activation. We conclude that

induced rDNA gene looping is a direct and rapid consequence of c-Myc activation that does not require induction of c-Myc target genes, including the rDNA. The results suggest a model in which c-Myc induced gene looping within the rDNA repeats forms part of the pathway by which rDNA transcription is induced when activated quiescent cells enter the growth cycle.

Quiescent cells contain essentially undetectable levels of c-Myc. Upon growth stimulation there is a rapid pulse of c-Myc expression that subsequently falls back to the low but detectable levels associated with growing cells [17,18]. It is therefore likely that c-Myc has some functions that are specific to the transient pulse in its expression when quiescent cells enter the cell cyle. Our results suggest that one such function could be establishing rDNA gene loops as part of a nucleolar reorganisation process that is required for the increased rate of ribosome biogenesis associated with growing cells. Nucleolar morphology is used as an important histological marker in the diagnosis and staging of tumours, since in some cancer types c-Myc overexpression is correlated with altered nucleolar morphology and poor prognosis [19,20]. It will thus be of key importance to further understand the role of c-Myc in nucleolar structure and function.

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AUTHOR CONTRIBUTIONS

CS performed all the experiments in the final paper. RB performed several experiments that were important for the final paper but were not included in it. All authors were involved in planning the work, data analysis and interpretation as well as writing of the final paper.

METHODS

Cell lines, culture conditions, and chemical treatments

Rat fibroblasts expressing MycER^{TM} (Rat1MycER)[21], the parental cell line Rat1$^{c\text{-}myc+/+}$ (clone TGR1), Rat1$^{c\text{-}myc-/-}$ (clone HO15.19)[22], and Smoxi 4 (H015.19-MycER)[23] were maintained in Dulbecco's modified Eagles's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 0.1% (v/v) gentamycin (Gibco) at 37 ^{o}C in a 5% CO_2 atmosphere. In starvation / refeeding experiments, cells were grown to confluence and starved in DMEM without FBS for 3 days, and then either left untreated or incubated with 200 nM 4-hydroxytamoxifen (4-HT) (Sigma) or DMEM containing 10% FBS for the indicated times. For Actinomycin D (Sigma) treatment, the final concentration of 1 μg/ml was added to culture medium one hour before 4-HT induction of c-Myc.

Northern blotting

Total cellular RNA was extracted from rat fibroblasts using the RNeasy Mini kit (QIAGEN) as recommended by the manufacturer. 10 µg of cellular RNA was resolved on 1% agarose gels containing formaldehyde (Sigma) (1 ml 37% stock/20 ml agarose gel), and transferred to Hybond N membrane (Amersham). Rat 45S pre-rRNA was detected using a radiolabelled probe that hybridizes to the 5'-terminal part (nucleotides 2-201) of rat rRNA. The radiolabelled probe was synthesized by Rediprime II random prime labeling system (Amersham). The hybridization images were captured and quantified using a FLA-3000 phosphoimager system (Fujifilm).

Chromatin immunoprecipitation (ChIP) assays

Preparation of nucleoli from formaldehyde-fixed cells and sonication of nucleolar chromatin were carried out as described previously[24] with little modification. The average length of sonicated nucleolar chromatin ranges from 500 bp to 1 kb. We used antibodies specific for Myc (N-262, sc-764), SL1 (TBP, N-12, sc-204), Polymerase I (RPA 194, N-16, sc-17916) (Santa Cruz Biotechnology). After overnight incubation at 4° C, 30 µl of a 50% slurry of salmon sperm DNA/protein G agarose beads (Upstate) was added, and the mixtures were rotated at 4 °C for 1 hour. The DNA-protein complexes were eluted twice from the beads with 250 µl of buffer containing 0.1M $NaHCO_3$ and 1% SDS. After reversal of the crosslinks by incubation at 65 °C overnight, proteinase K and RNase treatment, extraction with pheonol-chloroform, and ethanol precipitation, the recovered DNAs were resuspended in 200 ul of dH_2O for real-time PCR analysis.

Quantitative PCR assays

The sequences and locations of primer sets corresponding to rat ribosomal RNA are listed in Supplementary Table S1 online. DNA samples recovered from ChIP assays were used as templates in real-time PCR using a MyiQ™ single colour real-time PCR detection system (Bio-Rad), and samples from individual ChIP ssays were analyzed in triplicate. Each PCR mixture contained 5 pmol of each primer, 5µl (5/200) DNA templates in a final volume 25 µl of 1x iQ™ SYBRGreen Supermix (Bio-Rad). Input DNA was diluted 1:10 prior to analysis. The cycling program was set as follows: denature at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 30 seconds and 63 °C for 30 seconds and 72° C for 40 seconds, and then extension at 72° C for 7 minutes. The cycle threshold (CT) was set within the linear range of all PCR

reactions. The fold enrichment was calculated relative to the input DNA for each primer set.

Chromatin conformation capture assays

The procedure for chromosome conformation capture (3C) has been described in detail previously[13]. Briefly, cells were cross-linked using 2% formaldehyde (Sigma) at room temperature for 10 minutes. Cross-linking was stopped by addition of glycine at a final concentration of 0.125 M for 5 minutes. Cells were pelleted and intact nuclei were resuspended in nucleus lysis buffer (Sigma) containing protease inhibitors (Roche). The relative concentration of the chromatin solutions was estimated by isolating DNA from a sample and measurement of DNA concentration. Appropriate amounts of restriction enzymes *BamH*I, *Hind*III (New England Biolabs) (12 U/ μg DNA) were used for digestion overnight. After ligation, cross-linking was reversed and DNA extracted by phenol/chloroform prior to precipitation by ethanol. DNA pellets were dissolved in 100 μl of 10mM Tris (pH 8.0). Control templates for the 3C assay were generated by the following approaches: DNA fragments containing the *BamH*I or *Hind*III cutting site were amplified by PCR with a specific primer set (see Supplementary Table S2 online), purified by gel extraction, and then digested with restriction enzyme and ligated. For 3C analysis, primer sets and their corresponding locations are listed in Supplementary Table S3 online. The PCR conditions were standardized to 40 cycles of 95° C for 30 seconds, 61.1° C for 30 seconds, and 72° C for 40 seconds, followed by 72° C for 10 minutes.

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FIGURE LEGENDS

Figure 1. c-Myc is a direct inducer of rDNA transcription in rat cells. (a) Schematic representation of E-boxes and the location of amplified regions upstream and downstream of the rat rRNA coding regions that were used in chromatin immunoprecipitation (ChIP) assays. The sequence of the rat rDNA repeat was constructed from 7 GeneBank sequences (accession numbers: X04084, X03838, X61110, X00677, X16321, V01270, and X03695). The position of canonical (CACGTG) or non-canonical (CACATG, CATGTG, CACGCG) E-boxes were marked by vertical bars in relation to the 18S, 5.8S, 28S coding regions of the rDNA. The transcription start site is indicated by the bent arrow and T0 and T1-10 indicate binding sites for the transcription terminator factor (TTF-1). Regions amplified in ChIP experiments are shown by the horizontal bars, which are labeled to show their approximate position in relation to the transcription start site. **(b)** Serum induces c-Myc binding to E-box containing regions upstream and downstream of the rRNA coding regions. HO15.19 ($myc^{-/-}$) rat fibroblasts, or TGR1 ($myc^{+/+}$) cells were starved by growth without serum for three days, and either stimulated with serum for 6 hours or not stimulated. Chromatin was prepared from these cells for ChIP assays and real-time PCR to test for association of endogenous c-Myc upstream and downstream of the rDNA coding regions. The normalized binding shown is the mean of three independent ChIP assays (± standard error). **(c)** c-Myc mediates serum-induced rRNA transcription. Northern blot analysis of pre-rRNA levels in the quiescent TGR1 ($myc^{+/+}$) and HO15.19 ($myc^{-/-}$) cells before and after stimulation with serum for 6 hours. The 45S pre-rRNA was identified by a radio-labeled probe that is complementary to the first 201 nucleotides of rat rRNA transcripts. Ethidium-

bromide-stained 28S and 18S rRNA are shown as loading controls. (d) c-Myc accelerates rRNA synthesis in re-entry of starved cells into the growth cycle. Serum-starved Smoxi4 cells (*myc*$^{-/-}$, Myc/ER) were stimulated with serum or 4-hydroxytamoxifen (4-HT) for the indicated times. Other annotations are as in **c**.

Figure 2. **c-Myc activation leads to association of RNA Polymerase I and SL-1 with sequences downstream of the rDNA transcription unit. (a)** Serum feeding of starved cells leads to c-Myc dependent association of RNA Polymerase I (left panel) and SL-1 (right panel) upstream and downstream of the rRNA coding regions. ChIP assays and real-time PCR were performed to test for association of endogenous c-Myc upstream and downstream of the rDNA coding regions, using chromatin prepared from the serum-deprived HO15.19 cells (*myc*$^{-/-}$) with or without stimulation with serum for 6 hours and serum-deprived TGR1 cells (*myc*$^{+/+}$) with or without serum induction (6 hours). Annotation is as for Fig 1b. **(b)** c-Myc activation in starved cells is sufficient to cause increased association of RNA Polymerase I (left panel) and SL-1 (right panel) upstream and downstream of the rRNA coding regions. ChIP assays and real-time PCR were performed to test for association of endogenous c-Myc upstream and downstream of the rDNA coding regions, using chromatin prepared from the serum-deprived Rat1(Myc/ER) cells with or without induction of c-Myc with 4-HT (6 hours). Annotation is as for Fig 1b.

Figure 3. **rDNA regions upstream and downstream of the rDNA coding regions are juxtaposed in the nucleolar chromatin of growing cells. (a)** Schematic depiction of restriction enzyme cleavage sites and the position of primers used in the chromatin conformation capture (3C) assay. Vertical arrows above and below the

rDNA repeat show the locations of cleavage sites for *Bam*HI and *Hind*III, respectively. The location and directionality of primers are shown by horizontal arrows. The region amplified as a loading control for the 3C assays is shown (LC). Other annotations are as for Fig 1a. **(b)** A looped topological conformation of the rDNA is observed in growing cells such that regions upstream and downstream of the rRNA coding regions are juxtaposed. Results from 3C analysis with different primer pairs (Primer) of chromatin prepared from growing TGR1 ($myc^{+/+}$) cells by cross-linking or not with 2% formaldehyde, and digestion with *Hind* III (left panel) or *Bam*HI (right panel), followed by ligation or not. Positive 3C signals were dependent on both stabilization of chromatin conformation by cross-linking (FA) and ligation (ligase). Molecular weight markers are shown in lane M and lane C shows the size of the PCR fragment expected in the 3C assay if the tested DNA ends are in close proximity to each other (also shown by arrowheads). The loading control for each lane is shown (LC).

Figure 4. **c-Myc induces rapid formation of gene loops independently of rRNA transcription in the rDNA of cells as they enter the growth cycle. (a)** Gene looping of the rDNA is dependent on cell growth status and c-Myc. 3C assays performed on chromatin prepared from serum-deprived HO15.19 ($myc^{-/-}$) and TGR1 ($myc^{+/+}$) before and after serum stimulation. Annotations as for Fig 3b. **(b)** Specific activation of c-Myc in serum-starved cells is sufficient to induce gene looping independently of rDNA transcription. 3C assays performed on starved Rat1-MycER cells untreated or treated with 4-HT for 6 hours in the presence or absence of actinomycin-D (1 μg/ml), added 1 hour prior to 4-HT addition. Annotations as for Fig 3b. **(c)** rDNA transcription is effectively inhibited by Actinomycin D treatment. Northern blot using

RNA from starved Rat1-MycER cells untreated or treated with 4-HT for 6 hours in the presence or absence of actinomycin-D (1 μg/ml), added 1 hour prior to 4-HT addition. Annotations as for Fig 1c. **(d)** c-Myc activation induces rDNA gene loop formation rapidly in starved cells. 3C assays using starved Rat1MycER cells stimulated with 4-HT for the indicated times. Annotations as for Fig 3b.

Figure S1. The divergence of ribosomal DNA sequences between mammalian species. The human ribosomal DNA, derived from GeneBank no. U13369, contains transcribed region (5'ETS, 18S, ITS1, 5.8S, ITS2, 28S, 3'ETS) and un-transcribed region (IGS). The mouse rDNA sequence was retrieved from GeneBank no. BK000964, and the rat rDNA sequence was pieced together from GeneBank X04084, X03838, X61110, X00677, X16321, V01270, and X03695. The vertical bars represent the location of the potential canonical or non-canonical E boxes in mammalian rRNA genes. The alignment of rRNA genes between human, mouse, and rat was analyzed by Macvector™ 6.5.3 software (MacVector. Inc). The percentages shown are representative of homogeneity between human and mouse or rat rDNA.

Figure S2. c-Myc activates rRNA synthesis directly. **(a)** c-Myc induces rDNA transcription independent of polymerase II transcription. Northern blot analysis was performed by hybridizing the radiolabelled probe specific to pre-rRNA. Serum-starved Rat1MycER cells were stimulated with 4-HT or not for 6 hours, in the presence or absence of, α-amanitin (2 μg/ml) added one hour before 4-HT induction. Ethidium bromide-stained 28S and 18S are shown as loading controls. **(b)** Inhibition by α-amanitin of mRNA expression of the polymerase II transcribed c-Myc target cyclin D2. Total cellular RNA was isolated from serum-deprived Rat1MycER

fibroblasts stimulated with 4-HT or not for 6 hours. Total RNA was analysed by RT-PCR, using rat cyclin D2 (GeneBank no. NM 022267) primer pair: forward, TTACCTGGACCGTTTCTTGG (243-262); reverse, TGCTCAATGAAGTCGTGAGG (463-482), and rat 28S rRNA (GeneBank no. V01270) primer pair: forward, ACGGACCAAGGAGTCTAACGCGTG (5086-5109); reverse, CCAGAGTTTCCTCTGCTTCG (5313-5332).