Integrative analysis of RNA polymerase II and transcriptional dynamics upon MYC activation

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Overexpression of the MYC transcription factor causes its widespread interaction with regulatory elements in the genome but leads to the up- and down-regulation of discrete sets of genes. The molecular determinants of these selective transcriptional responses remain elusive. Here, we present an integrated time-course analysis of transcription and mRNA dynamics following MYC activation in proliferating mouse fibroblasts, based on chromatin immunoprecipitation, metabolic labeling of newly synthesized RNA, extensive sequencing, and mathematical modeling. Transcriptional activation correlated with the highest increases in MYC binding at promoters. Repression followed a reciprocal scenario, with the lowest gains in MYC binding. Altogether, the relative abundance (henceforth, “share”) of MYC at promoters was the strongest predictor of transcriptional responses in diverse cell types, predominating over MYC’s association with the corepressor ZBTB17 (also known as MIZ1). MYC activation elicited immediate loading of RNA polymerase II (RNAPII) at activated promoters, followed by increases in pause-release, while repressed promoters showed opposite effects. Gains and losses in RNAPII loading were proportional to the changes in the MYC share, suggesting that repression by MYC may be partly indirect, owing to competition for limiting amounts of RNAPII. Secondary to the changes in RNAPII loading, the dynamics of elongation and pre-mRNA processing were also rapidly altered at MYC regulated genes, leading to the transient accumulation of partially or aberrantly processed mRNAs. Altogether, our results shed light on how overexpressed MYC alters the various phases of the RNAPII cycle and the resulting transcriptional response.

[Supplemental material is available for this article.]
Results

Relationship between MYC binding and gene regulation

To address how binding of MYC to promoters affects gene regulation, we exploited a series of ChIP- and RNA-seq data sets with activated MYC and control samples. The first three were derived from in vitro models, including the post-translational activation of a Myc-ER chimera in mouse 3T9MYC-ER fibroblasts (Sabò et al. 2014), as well as the conditional expression of recombinant tetr-MYC protein in human osteosarcoma (U2OStet-MYC) (Walz et al. 2014) and B-cell lines (P493-6) (Lin et al. 2012). Two other data sets were based on tumors arising in MYC-transgenic mice, including Eµ-myc lymphomas (Sabò et al. 2014) and tet-MYC liver carcinomas (Kress et al. 2016), each confronted with its normal tissue counterpart (Supplemental Table S1).

Previous analyses in U2OStet-MYC cells indicated that the extent of either activation or repression by overexpressed MYC correlated with the increase in MYC occupancy at promoters (Walz et al. 2014). Our re-analysis of the same data confirmed this observation, whether performed as originally described (i.e., by binning activated and repressed mRNAs) (Supplemental Fig. S1A, top) or considering every differentially expressed transcript (Fig. 1A).

The four other model systems confirmed the positive correlation between gene activation and binding (Fig. 1A; Supplemental Fig. S1A, red lines) but showed the opposite for repressed genes (blue lines): as a consequence, the whole transcriptome showed a more homogeneous trend, repressed and activated genes showing the lowest and highest gains in MYC binding, respectively (Fig. 1A, orange lines). This relationship was reinforced by calculating the “share” of MYC at each promoter, obtained by normalizing each binding event by the total amount of MYC associated with the...
genome. The share concept was adopted to focus on the changes in binding that were emerging from the global changes (which were normalized out). This concept allowed us to define two classes of promoters with either increased or decreased shares following MYC activation (Fig. 1B): remarkably, the threshold separating these two classes (Fig. 1A, dashed vertical lines) identified the amount of MYC binding that optimally separated transcriptionally activated from repressed genes in each model system (Fig. 1A,C). This effect was further reinforced when distinguishing primary (MYC-dependent) from secondary (independent) regulatory events in tet-MYC liver tumors (Supplemental Fig. S1B,C; Kress et al. 2016).

The apparently opposite regulatory behavior of repressed genes in U2OS cells is a paradoxical result, which remains to be explained. We deem it unlikely, however, for this to represent a real distinctive feature of these cells. Indeed, we must note that the negative correlation between the changes in MYC share and transcription of repressed genes in the U2OS data set was remarkably low and not statistically significant, whether analyzing genes individually (Spearman correlation −0.03, P = 0.12) (Fig. 1A) or binned (−0.05, P = 0.81) (Supplemental Fig. S1A). The other data sets, instead, all gave robust positive and statistically significant correlations. The reasons for this discrepancy may reside in a number of technical parameters, the resolution of which is beyond the scope of our work.

In U2OStet-MYC cells, the transcriptional response to MYC has also been correlated with the MYC/ZBTB17 ratio at promoters (Lorenzin et al. 2016): indeed, we confirmed that the MYC/ZBTB17 ratio and the variation in the MYC share were complementary predictors of gene expression changes (Supplemental Fig. S1E). In 3TgMYC-ER cells and Eµ-myc lymphomas, instead, the MYC share had much stronger predictive values, with minor albeit significant contributions of ZBTB17—whether expressed as a MYC/ZBTB17 ratio or as straight ZBTB17 binding (Supplemental Fig. S1E). In particular, the ability of MYC to recruit RNAPII seemed to be fine-tuned by ZBTB17: indeed, in all the systems analyzed, the gain in MYC binding that discriminated between activation and repression slightly increased with the level of ZBTB17 binding (Supplemental Fig. S1F). Overall, the above observations show that the variation in MYC share at promoters was the main feature separating induced from repressed genes across cell types: gains in MYC binding that were emerging from the global changes (which were normalized out). This concept allowed us to define two classes of promoters with either increased or decreased shares following MYC activation (Supplemental Fig. S1E). In 3T9MYC-ER cells and Eµ-tet-MYC liver tumors (Supplemental Fig. S1B,C; Kress et al. 2016).

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with this concept, MYC activation resulted in increased pause-release at activated promoters (clusters c1–4, with the exception of c5) (Fig. 3C; Supplemental Fig. S3B). However, these changes were associated with sudden and more prominent changes in RNAiPII loading (Fig. 3A,C). Following this initial burst, recruitment rates were stabilized at higher levels compared to the untreated condition (Supplemental Fig. S3B). We conclude that MYC promotes both RNAiPII loading and pause-release, thus regulating two key steps in transcription initiation (Jonkers and Li 2015).

In parallel with the above, down-regulated clusters (c7–12, with the exception of c6) showed marked decreases in the RNAiPII signal at promoters (Fig. 3A), owing primarily to decreased loading (p1) (Fig. 3C; Supplemental Fig. S3B). In four clusters (c9–12), p1 values transiently became negative, indicating nonproductive detachment of RNAiPII from promoters. Importantly, the reduced levels of RNAiPII were not due to increased pause release, as p2 values also rapidly went down in response to MYC activation (with the exception of c7). Thus, MYC-repressed genes underwent a general reduction in RNAiPII loading.

We showed above that changes in MYC share at promoters correlated with changes in gene expression (Fig. 1) and synthesis rates (Fig. 2C,E). Logically, RNAiPII flux at promoters also followed the MYC share (Fig. 3E) and was sufficient to explain most of the variation in polymerase occupancy and synthesis rate (p1, on average 69% of explained variance) (Fig. 3D), while p2, p3, and p4 showed lesser relevance (10%, 34%, and 17%, respectively). Hence, the first effect of MYC-ER was a rapid modulation of RNAiPII flux at promoters, with increases in recruitment at activated genes and, reciprocally, decreases—or even inversion—at repressed loci, which also showed the lowest gains in MYC binding. Altogether, the above observations show that acute activation of MYC causes direct recruitment of RNAiPII to activated promoters, accounting for most of the observed changes in RNA synthesis, at either up- or down-regulated loci.

**Figure 2.** Dynamics of mRNA synthesis, processing, and degradation. (A) Study design indicating the collected HTS data, the time points of OHT treatment, and the number of replicates. In 4sU-seq samples, 4sU was added to the culture medium for 10 min prior to collection at every time point, to label and purify newly synthesized RNA. (B) Schematic representation of the kinetic rates (in red in the figure) of transcriptional regulation: immature and mature mRNA abundances, as well as synthesis rates, were derived directly from the experimental data, taking advantage of exonic and intronic reads in total and nascent RNA-seq data. Conversely, processing and degradation rates (in italic in the figure) were inferred from the integrated analysis of these data based on mathematical modeling. (C) Hierarchical clustering of the transcriptional response and change of MYC share for MYC-bound differentially expressed genes. Genes and time points are depicted in the rows and columns, respectively, and up- (red) or down- (blue) modulation is determined as the log2 ratio to the untreated condition. (D) Principal component analysis of the transcriptional response depicted in C; for each data type, subsequent time points of OHT treatment follow from light to darker shading; PC1 and PC2 are the first and second principal components, accounting for 80% and 9% of the explained variance, respectively. (E) Density scatter plot (darker colors for higher density) of the variation of MYC ChIP-seq signal within promoters (x-axis) vs. the corresponding change in synthesis rate (y-axis). The red, blue, and orange lines capture the trend for induced, repressed, and both (combined) set of genes, respectively. For the same set of genes, the Spearman correlation is reported.

**Dynamics of RNAiPII elongation and pre-mRNA processing**

The above observations revealed a paradox, in that changes in synthesis rate were delayed relative to the variations in RNAiPII density at gene-bodies. In particular, RNAiPII accumulated within induced genes and, reciprocally, was already lost from repressed genes 10 min after MYC-ER activation (Fig. 3A), originating at the 5′ end of transcriptional units (Supplemental Fig. S4A). Notably, at induced loci, the increases in RNAiPII density exceeded those in RNA synthesis (Supplemental Fig. S3C), suggesting that decreasing rates of polymerase elongation contributed to RNAiPII accumulation in induced genes (Ehrensberger et al. 2013), as confirmed by our modeling (Fig. 3C). Indeed, fixing elongation rates in silico at the levels of the untreated condition was detrimental for the ability of the model to predict either RNAiPII dynamics at gene-bodies or synthesis rates (Supplemental Fig. S4B). Moreover, while MYC-induced increases in pause-release
also contributed to RNAPII accumulation in induced genes, the changes in elongation were more sustained (Fig. 3C) and contributed a larger part of the variance (p2 vs. p3) (Fig. 3D). This situation was mirrored at repressed loci, at which changes in p2 and p3 determined a reduced density of RNAPII at the gene-body, especially for the strongest responders (cl9–12) (Fig. 3A,C).

Shortly after the decrease in RNAPII elongation, almost half of the induced genes showed decreasing rates of pre-mRNA processing with concomitant accumulation of pre-mRNA, anticipating the transcriptional response (Fig. 4; Supplemental Fig. S4C). Opposite effects occurred at a subset of repressed genes. In silico modeling confirmed that fixing the processing rates of the induced genes at the levels of the untreated condition prevented the observed accumulation of pre-mRNA (Supplemental Fig. S4D,E).

Consistent with previous observations (Dujardin et al. 2014), the observed changes in RNAPII elongation were associated also with alterations in splicing affecting exon incorporation or intron retention at several hundred loci (Supplemental Fig. S4G–I). These alterations peaked at the time of maximum changes in the rates of elongation and processing (30 min and 1 h for induced and repressed, respectively) and were enriched at the 3′ end of genes (Supplemental Fig. S4G,1). Additional alterations occurred later, possibly due to the downstream action of splicing factors, which were enriched among MYC-regulated gene products.

**Discussion**

Altogether, our analysis reveals several key features of the transcriptional alterations brought about by MYC overexpression in various systems. First, we quantified the ability of MYC binding to predict the transcriptional response of its target genes, revealing that the variation in the proportion of MYC bound at promoters (MYC share) could effectively discriminate induced from repressed genes. The role of the MYC share in directing the induction or repression of MYC target genes emerged as a common denominator among these systems, strengthening the view that transcriptional amplification, when observed, does not occur as a direct effect of MYC but rather as a secondary consequence of cellular activation (Kress et al. 2016).

Second, we dissected the effects of MYC on the RNAPII life cycle along transcriptional units, identifying loading as the key regulated step. Previous reports indicated that MYC activates transcription through RNAPII pause-release (Eberhardy and Farnham 2002; Bouchard et al. 2004; Rahl et al. 2010); while we confirmed this effect at MYC-activated genes, pause-release was secondary to...
RNAPII loading. It is noteworthy here that we are not the first to report that MYC binding leads to increased RNAPII at promoters (Walz et al. 2014): our modeling, however, allowed us to directly quantify this step, overcoming the limitations of using solely ChIP-seq density data (Ehrensberger et al. 2013).

Third, transcriptional repression was associated both with the lowest gains in MYC binding and with decreased RNAPII recruitment. As the amount of chromatin-bound RNAPII remained relatively stable following MYC activation (Supplemental Fig. S3E), loss of RNAPII from repressed promoters may have largely resulted from competition for limiting amounts of polymerase. In particular, a role for the latter is supported by our data, since pre-RNAPIA processing rates decreased in parallel to the increase of RNAPII at gene-bodies, leading to the accumulation of unprocessed mRNAs. This points to a limiting activity of the spliceosomal machinery at MYC-activated loci, consistent with its critical role for cell survival in MYC-driven tumors (Hsu et al. 2015; Koh et al. 2015).

Methods

Cell culture

3T3MYC-ER fibroblasts were obtained by infecting 3T3 c-Myc flox/flox immortalized fibroblasts (Trumpp et al. 2001) with a pBabe-Bleo retrovirus encoding the MYC-ER chimera (Sabò et al. 2014) and were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin/streptomycin. These cells are not listed in any database of commonly misidentified cells and are negative for mycoplasma. Cells were used at sub-confluent cell densities for all experiments. Due to infection with the pBabe bleomycin MYC-ER construct, cells are resistant for zeocin. Upon thawing, cells were maintained for 7–10 d in zeocin-containing medium (100 µg/mL) but grown without zeocin for subsequent experiments. MYC-ER activation was achieved by addition of 400 nM of the synthetic 4-hydroxytamoxifen (OHT; Sigma-Aldrich). For RNA degradation validation, cells were treated with 1 µM flavopiridol hydrochloride hydrate (Sigma-Aldrich) for the indicated time points.

4sU RNA-seq

Detection of nascent RNA by metabolic labeling using 4sU (4-methio-uridine) has been performed as described before (Sabò et al. 2014) by labeling the cells with 300 mM 4sU for 10 min. The 4sU-sequencing libraries were prepared with the TruSeq RNA Sample Prep kit v2 (Illumina) following the manufacturer’s instructions starting from the RNA fragmentation step.

Data access

The high-throughput sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE98420. The complete source code for all analyses, including intermediary results and the raw images, is available in the Supplemental Material.
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Author contributions: S.d.P., T.R.K., B.A., and M.P. conceived the work, designed the experiments, and interpreted the data. S.d.P., B.A., and M.P. supervised the project and wrote the manuscript. T.R.K., A.S., C.L., A.V., M.D., and S.C. performed experiments, and S.d.P. and M.J.M. performed computational data analysis.

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