N-Myc Regulates a Widespread Euchromatic Program in the Human Genome Partially Independent of Its Role as a Classical Transcription Factor

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Abstract

Myc proteins have long been modeled to operate strictly as classic gene-specific transcription factors; however, we find that N-Myc has a robust role in the human genome in regulating global cellular euchromatin, including that of intergenic regions. Strikingly, 90% to 95% of the total genomic euchromatic marks histone H3 acetylated at lysine 9 and methylated at lysine 4 is N-Myc–dependent. However, Myc regulation of transcription, even of genes it directly binds and at which it is required for the maintenance of active chromatin, is generally weak. Thus, Myc has a much more potent ability to regulate large domains of euchromatin than to influence the transcription of individual genes. Overall, Myc regulation of chromatin in the human genome includes both specific genes, but also expansive genomic domains that invoke functions independent of a classic transcription factor. These findings support a new dual model for Myc chromatin function with important implications for the role of Myc in cancer and stem cell biology, including that of induced pluripotent stem cells. [Cancer Res 2008;68(23):9654–62]

Introduction

Myc proteins belong to the basic helix-loop-helix zipper superfamily of DNA-binding proteins that dimerize on CANNTG E-box sequences to function as transcription factors (TF). Myc forms dimers with Max on the CACGTG sequence that activate transcription (1–3), whereas Myc also has a repressive function as well through association with Miz-1 (4). Myc proteins regulate many aspects of embryonic development as well as normal cell biology including cell cycle, metabolism, differentiation, senescence, apoptosis, and DNA replication (reviewed in ref. 5). Deregulation of *Myc* genes, particularly N-Myc, has been strongly linked with many human neural cancers including neuroblastoma and medulloblastoma (6–8). In medulloblastoma, Myc can cooperate with REST to drive tumorigenesis by blocking differentiation (9), which may be a key function in normal stem cells as well. Given the roles of both Myc and REST in embryonic stem cell (ESC) biology (10), they may also cooperate in that context. N-Myc amplification is known to correlate with poor prognosis in neuroblastoma (11), but the molecular mechanism by which N-Myc contributes to tumorigenesis is still largely an open question. Myc proteins are atypical basic helix-loop-helix zipper factors in that they are relatively weak transcriptional activators of potentially thousands of genes (12). This weak, but apparently widespread, transcriptional function is a long-standing puzzle.

Intense interest in Myc function has been stimulated more recently by studies linking both c-Myc and N-Myc to the generation of induced pluripotent stem (iPS) cells (reviewed in refs. 13, 14) as well as to murine ESC biology (15), together suggesting that Myc has important roles in regulating stem cell self-renewal and pluripotency. The molecular mechanisms by which Myc influences iPS cell formation and ESC biology are open questions. One possibility is that Myc contributes to the process through global chromatin reprogramming, suggested by studies (16, 17) implicating Myc in the regulation of widespread histone modifications. Consistent with this idea, a very large Myc-regulated transcriptional program is operative in ESC that may have significance for Myc's normal and neoplastic functions (18, 19) as well as iPS formation, but how Myc regulates this program at the chromatin level and its effect on cell biology remain unknown.

In neural stem cells, loss of N-Myc is sufficient to cause nuclear condensation, most likely due to a global spread of heterochromatin (20). Myc's recruitment of histone acetyltransferases such as GCN5 (21) and TIP60 (22), as well as its regulation of histone acetylation at a number of genic loci (17, 23, 24), suggests that the regulation of euchromatin through histone acetylation is involved. Unlike most TFs, Myc binds to tens of thousands of genomic sites, both near and far from core promoters (24–29), indicating that the euchromatic program may be quite expansive. Also, at least in the genes, Myc may directly affect transcription via binding to PTEF-b (30, 31). Additional evidence suggests that the Myc-regulated chromatin program involves both histone acetylation but also methylation of lysine 4 of histone H3 (triMeK4; ref. 16), possibly through the demethylase LID (32).

Because Myc's normal and neoplastic functions depend on its ability to bind DNA and likely in turn to influence chromatin, the specific nature of its activity on cellular chromatin is a critically important open question. To address this key gap, we conducted a functional genomics study in which we mapped Myc binding and chromatin function in a global, unbiased manner in the human genome using a conditional N-Myc transgene in neuroblastoma cells, finding that Myc has a global role in euchromatin maintenance. These findings support a new model in which Myc proteins act not only as classic TF but also more broadly to maintain widespread euchromatin.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Expression microarray studies. RNA samples were prepared from TET21N cells in biological duplicates for expression microarray studies. WG-6 beadchip arrays from Illumina were used. Data was normalized and analyzed using Illumina Beadstudio 3.0 and GeneSpringGX 7.3.1 (Agilent Technologies).

Chromatin immunoprecipitation-chip. Chromatin samples were prepared and PCR conducted as previously described (33); some PCRs initially failed, in which case, a PCR enhancer mix (34) was used, but in all cases, the same PCRs were used for different immunoprecipitations to be compared at the same gene. For TET21N cells, 4 × 15 cm plates ($\sim 10^8$ cells) were crosslinked per experiment. Antibodies used for chromatin immunoprecipitations (ChIP) include the following: N-Myc (1 μg/mL; Abcam), AcK9 (2.5 μg/mL; Upstate), and triMeK4 (2.5 μg/mL; Upstate). Rabbit IgG (2 μg) was used as a background control. Immunoprecipitated chromatin fragments were amplified using the Whole Genome Amplification kit (Sigma) for 2 × 14 cycles, purified, then checked for enrichment over control IgG and total samples before sending for probing of the ENCODE array (NimbleGen Systems).

Chromatin samples were used to probe ENCODE arrays and bioinformatics performed as previously described (28, 33). Briefly, for identification of the N-Myc binding sites on the ENCODE arrays which represent 1% of the human genome and include ~400 genes and intergenic regions, we used the Tamalpais program (28) and chose the L1 set of high confidence peaks for further analysis. Briefly, binding sites are identified as peaks that have a minimum of six consecutive probes in the top 2% of all probes on the array with a $P > 0.0001$. The identified binding sites were then determined to locate to a nearest gene based on GENCODE database (35). Each binding site was then classified as downstream or upstream relative to the distance of an annotated gene in GENCODE. Straight ChIP assays for verification were done using 10 ng of input amplicon DNA.

Manual peak counting was conducted by using SignalMap zoomed in to a high enough magnification to count individual peaks per cluster. Thresholds for scoring peaks were determined by the average peak intensity within a cluster at day 0 for a given ChIP. Log 2 value thresholds used were the following for N-Myc, AcK9, and triMeK4, respectively: Leng 9 (>1.5, 1.5, and 1.4), Luc7L (>1.0, 2.5, and 2.5), and ZNF259 (>1.3, 2.3, and 1.7).

Motif analysis. The peaks identified from the N-Myc ENCODE array were used to *de novo* identify putative N-Myc binding motifs and possible binding partner motifs using our previously developed method ChIPMotifs (36). Briefly, the ChIPMotifs approach incorporates a statistical bootstrap resampling method to identify the top motifs detected from a set of ChIP-chip training data using *ab initio* motif-finding programs such as Weeder (37) and MEME (38).

Immunoblotting and staining. Immunoblotting and staining were performed as previously described (16).

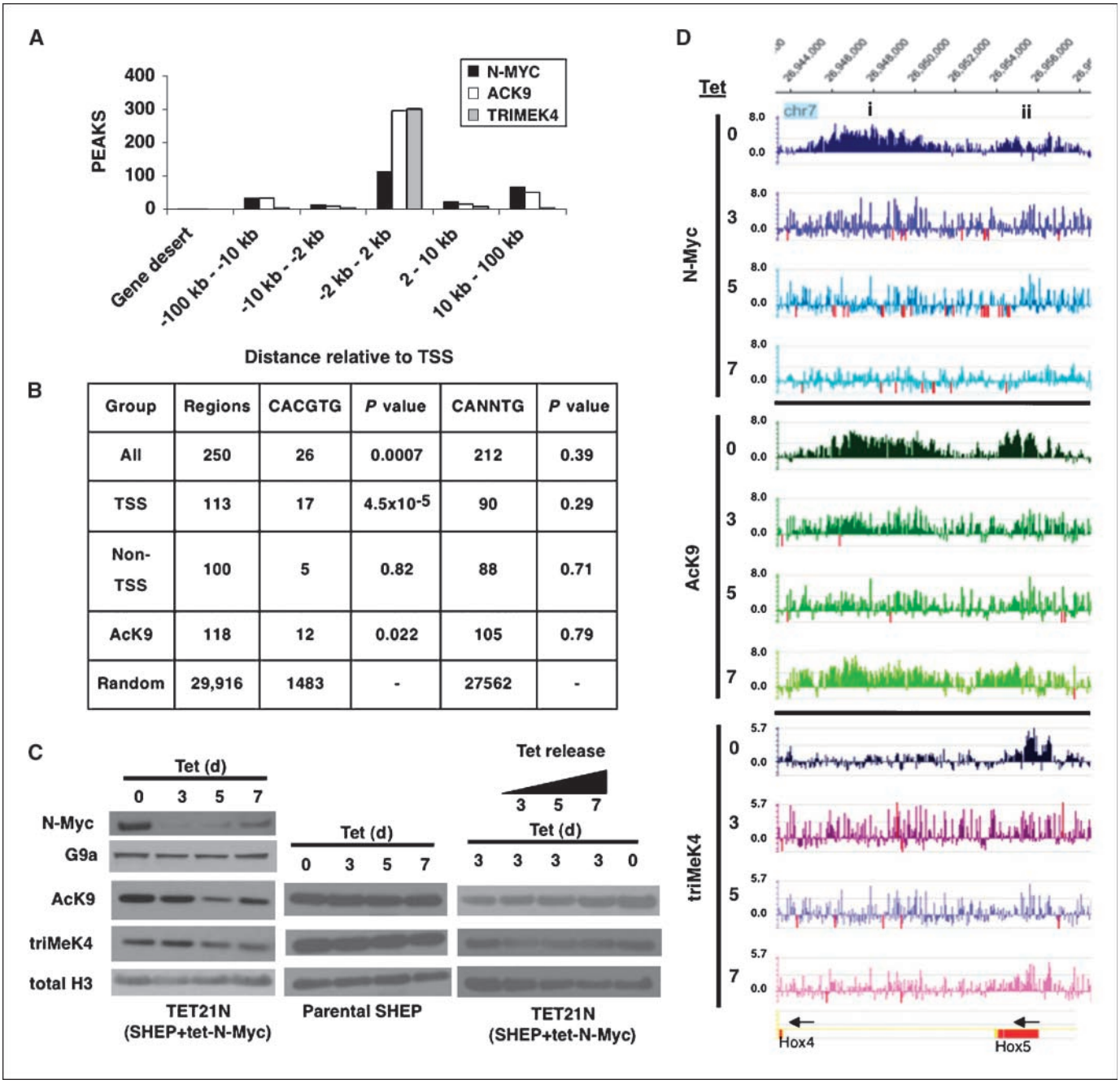
Results

N-Myc genomic binding is widespread, including domains both near and distant from promoters. N-Myc regulation of chromatin in the human genome was analyzed using a well-characterized human neuroblastoma cell line containing a tetracycline (Tet) repressible N-Myc transgene (TET21N; ref. 39). A functional genomics analysis was conducted on N-Myc binding in TET21N cells untreated (day 0) as well as treated with Tet for 3, 5, and 7 days using ChIP-chip with ENCODE arrays (NimbleGen Systems) covering 1% of the human genome (35). N-Myc binding in the day 0 control cells (Fig. 1*A*) was confirmed as specific by comparison to ChIP-chip conducted on cells treated for 3 days with Tet, in which N-Myc protein was absent. N-Myc bound a total of 250 sites in control (day 0) cells, whereas notably only 3 N-Myc binding sites were evident in cells treated with 3 days of Tet, with both scored at the same, high statistical stringency (L1; ref. 28). These data show that in this system, a strong reduction of N-Myc levels translates to essentially a complete absence of detectable N-Myc binding to chromatin. Even with peak scoring at the much lower L3 stringency, 397 binding sites were observed in the control cells and only 28 sites in day 3 cells. Extrapolated to the whole human genome, these findings predict ~25,000 to 40,000 total N-Myc binding sites with almost 40% of the binding to remote regions at least 10 kb from the transcriptional start sites (TSS; Fig. 1*A*), very similar to previous predictions for c-Myc (27, 28).

Evidence that N-Myc binding in nonpromoter regions is E-box–independent. We used the ChIPMotifs software (36) to directly scan for the Myc canonical E-Box motif (CACGTG) and a more degenerate E-Box (CANNTG) within the entire set of 250 N-Myc binding regions (Fig. 1*B*). CACGTG E-boxes were enriched in the data set of 250 N-Myc–bound sequences ($P = 0.0007$), whereas in contrast, the degenerate E-box was not significantly enriched ($P = 0.39$) here nor in any data set, indicating that binding to noncanonical E-boxes is not a common event. The Myc E-box CACGTG was most highly enriched specifically in TSS regions, with 17 of 113 binding regions having at least one E-Box, with a $P = 0.000045$ value (Fig. 1*B*). Intriguingly, whereas N-Myc binding to non-TSS by ChIP-chip seems to be just as robust as binding to TSS regions, the CACGTG E-box was not enriched in non-TSS regions as only 5 of 100 binding regions had the CACGTG E-Box ($P = 0.82$). This finding suggests that regulation of non-TSS regions by N-Myc might use a distinct method of binding DNA, possibly via a cofactor. We also found enrichment for CACGTG E-boxes in AcK9 regions with 12 of 118 binding regions having an E-Box ($P = 0.022$).

N-Myc genomic binding is strongly linked to AcK9 and triMeK4. In parallel to N-Myc, we also used ChIP-chip to determine the global distribution of AcK9 and triMeK4 in TET21N (Fig. 1*A*). The majority (92%) of triMeK4 was within TSS regions (−2 to +2 kb) as expected from previous studies (40). Although AcK9 was most abundant in TSS regions, it also had substantial binding (28%) elsewhere and the relative distribution of AcK9 peaks in regions progressively further from TSS were quite similar to that of N-Myc (Fig. 1*A*). Furthermore, in specific domains bound by N-Myc, peaks of binding for N-Myc, AcK9, and triMeK4 were most often remarkably similar in patterns and precise locations (Fig. 1*D*).

Loss of N-Myc decreases total cellular pools of two key active euchromatin marks in human neuroblastoma. By immunoblotting, Tet treatment of TET21N led to a pronounced global decrease of total acid-extractable AcK9 that correlated with decreased levels of N-Myc protein in the nucleus, most prominently at 5 days (Fig. 1*C, left*) with similar, but more modest, changes in triMeK4 also evident. It is notable that at 7 days, N-Myc levels again increased modestly due to the exhaustion of Tet in the medium. The global decreases in AcK9 with N-Myc depletion were partially reversed at day 7 with re-increased N-Myc levels. Tet alone seems to have no effect on global chromatin as the parental SHEP neuroblastoma cells, from which the TET21N were derived, show no changes in global chromatin with the same course of Tet treatment (Fig. 1*C, middle*). This finding also suggests that the effects on chromatin observed in TET21N cells are specific to loss of N-Myc. Treating cells with Tet for 3 days, which reduced AcK9 levels, followed by a switch to Tet-free medium for 3, 5, and 7 days (release) led to a progressive but only partial restoration of AcK9 even at day 7 of release (Fig. 1*C, right*). These data suggest that N-Myc may have the ability to establish domains of AcK9 as well as maintain AcK9 levels, but indicate that maintenance of AcK9 may be the predominant function.



Group	Regions	CACGTG	*P* value	CANNTG	*P* value
All	250	26	0.0007	212	0.39
TSS	113	17	4.5×10^{-5}	90	0.29
Non-TSS	100	5	0.82	88	0.71
AcK9	118	12	0.022	105	0.79
Random	29,916	1483	-	27562	-

Figure 1. Myc chromatin binding and regulation in human neuroblastoma cells. *A,* summary of ChIP-chip results on global distribution of N-Myc, AcK9, and triMeK4 in the human neuroblastoma genome. *B,* E-box enrichment in ChIP-chip data sets with *P* values calculated using the Fisher test to compare specific N-Myc data set groups to the random ENCODE data set. *C,* repression of N-Myc levels and total acid extractable active histone modifications with Tet treatment of Tet21N neuroblastoma cells (*left*). Immunoblots of nuclear (*top two rows*) and acid extracts (*bottom three rows*) of Tet21N cells treated with Tet for 0 to 7 days. Tet treatment does not influence AcK9 and triMeK4 in parental SHEP cells (*middle*). Tet treatment followed by ''release'' in Tet-free medium for 3, 5, and 7 days leads to a partial recovery of AcK9 (*right*). *D,* examples of N-Myc–bound domains. *Blue peaks,* N-Myc binding; *green peaks,* AcK9; *purple peaks,* triMeK4; and *red peaks,* off-scale. *Arrows,* direction of gene transcription of the indicated genes at the bottom. Domains (*i*) and (*ii*) are regions of widespread and focal binding/function, respectively.

N-Myc is essential for global maintenance of triMeK4 and AcK9 in both genic and intergenic regions. We theorized that there were two possible explanations for the tight association of N-Myc genomic binding with AcK9/triMeK4 peaks: (*a*) N-Myc binding depended on the presence of pre-existing AcK9 and triMeK4 as previously reported (17), or (*b*) alternatively, or in addition, that N-Myc played a key role in the establishment or maintenance of these histone marks. To test these possibilities, ChIP-chip was also performed for N-Myc, AcK9, and triMeK4 in TET21N cells treated with Tet for 3, 5, and 7 days (Fig. 1*D*). At days 3 and 5 of N-Myc depletion, nearly all AcK9 and triMeK4 peaks, irrespective of relative location to TSS, were lost. Interestingly at day 7, with the partial restoration of N-Myc levels, the losses of AcK9 and triMeK4 domains were partially reversed, apparently at the same locations and with the same patterns originally mapped at day 0 (Fig. 1*D*). Although detectable N-Myc binding was not restored at

most genomic sites at day 7, the rescue of AcK9 and triMeK4 at this time strongly suggests that N-Myc is acting on chromatin but with an affinity below that detectable in our assays. Some domains of N-Myc binding and regulation of AcK9/triMeK4 were expansive (10 kb or larger in size), whereas others were quite focal (Fig. 1*D*, regions *i* and *ii*; Fig. S1).

Global bioinformatics analysis of the ChIP-chip data using the Tamalpais program (28) indicates that after 3 and 5 days of N-Myc depletion, 87% and 90% of total genomic AcK9 sites were lost, respectively (Fig. 2*A*). In parallel, >93% and 86% of total genomic triMeK4 were lost (Fig. 2*B*). Thus, just a 3-day deficit of N-Myc was sufficient to induce a nearly complete and global loss of two key euchromatic histone modifications with the loss persisting up to 5 days. Some AcK9 and triMeK4 peaks, even those directly bound by N-Myc, were not detectably affected by N-Myc loss at days 3 and 5 (Fig. S2) and were still scored as peaks at L1 stringency, demonstrating that the 3-day and 5-day arrays were successfully probed and that loss of AcK9 and triMeK4 was specific to certain domains despite the overall profound losses of AcK9 and triMeK4. When AcK9 and triMeK4 binding were analyzed at lower stringencies (L1–L4, from highest to lowest; Table S1), we did not find a shift toward lower stringency peaks with loss of N-Myc. However, it seems some bona fide regulated domains remain at day 5 that are likely of even lower affinity than L4.

Evidence for both direct and indirect chromatin functions for N-Myc. Although N-Myc maintains 90% or more of total

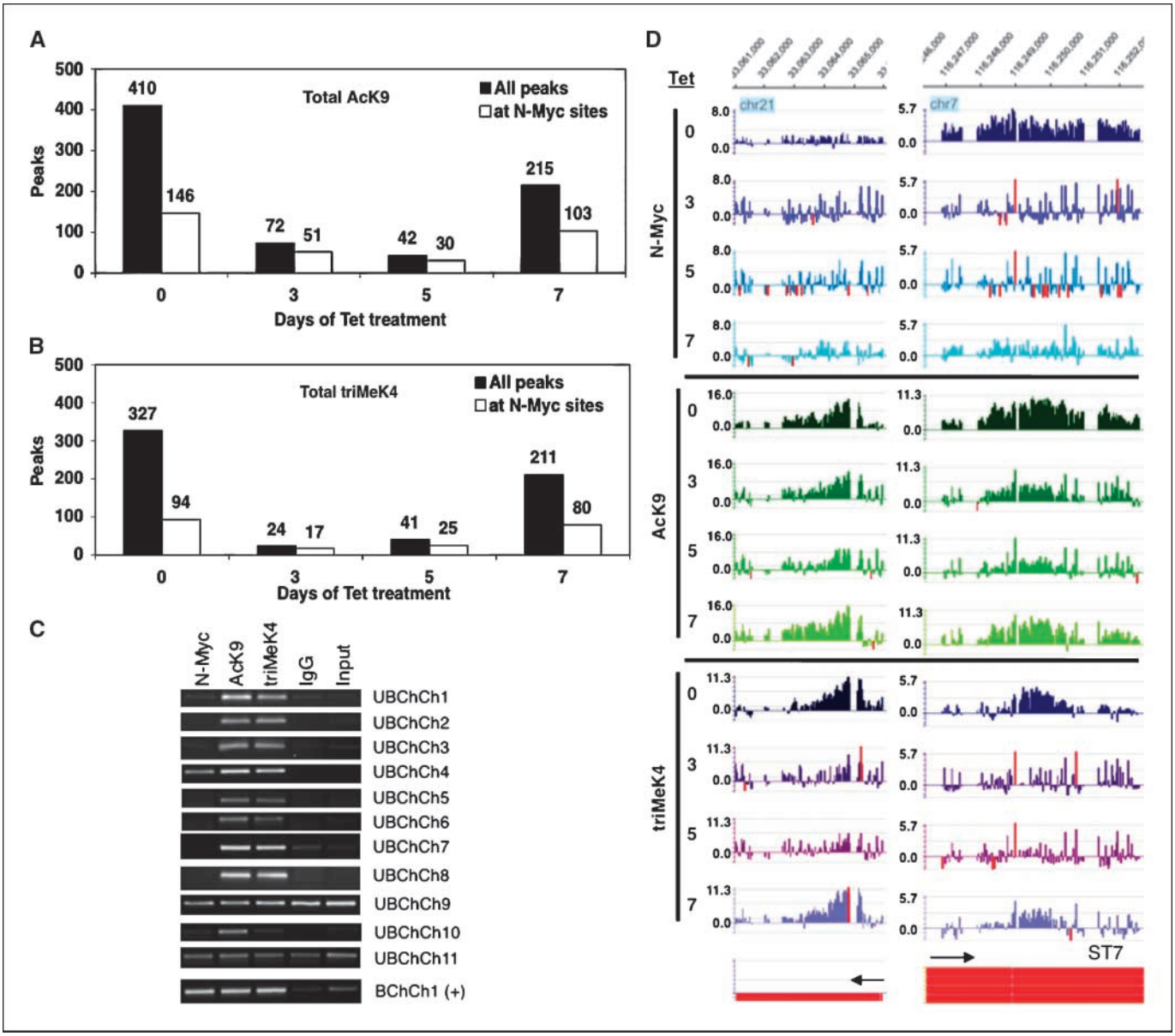


Figure 2. ChIP-chip indicates N-Myc is essential for global maintenance of AcK9 and triMeK4 throughout the human neuroblastoma genome including genic and nongenic regions. Total AcK9 (*A*) and triMeK4 (*B*) peaks at 0 to 7 d of Tet treatment. *C,* 11 regions that had values of <2-fold enrichment for N-Myc peaks (unbound by ChIP-chip; UBChCh) but had AcK9 and triMeK4 sensitive to N-Myc reduction were tested for potential false-negative N-Myc binding status by straight ChIP assays on control day 0 Tet21N amplicons. A strongly N-Myc–bound region, >4-fold enrichment, was included in parallel as a positive control, BChCh1(+). *D,* example of N-Myc–dependent AcK9 and triMeK4 domains unbound and bound by N-Myc (*left* and *right*). Gene on bottom left is not yet annotated.

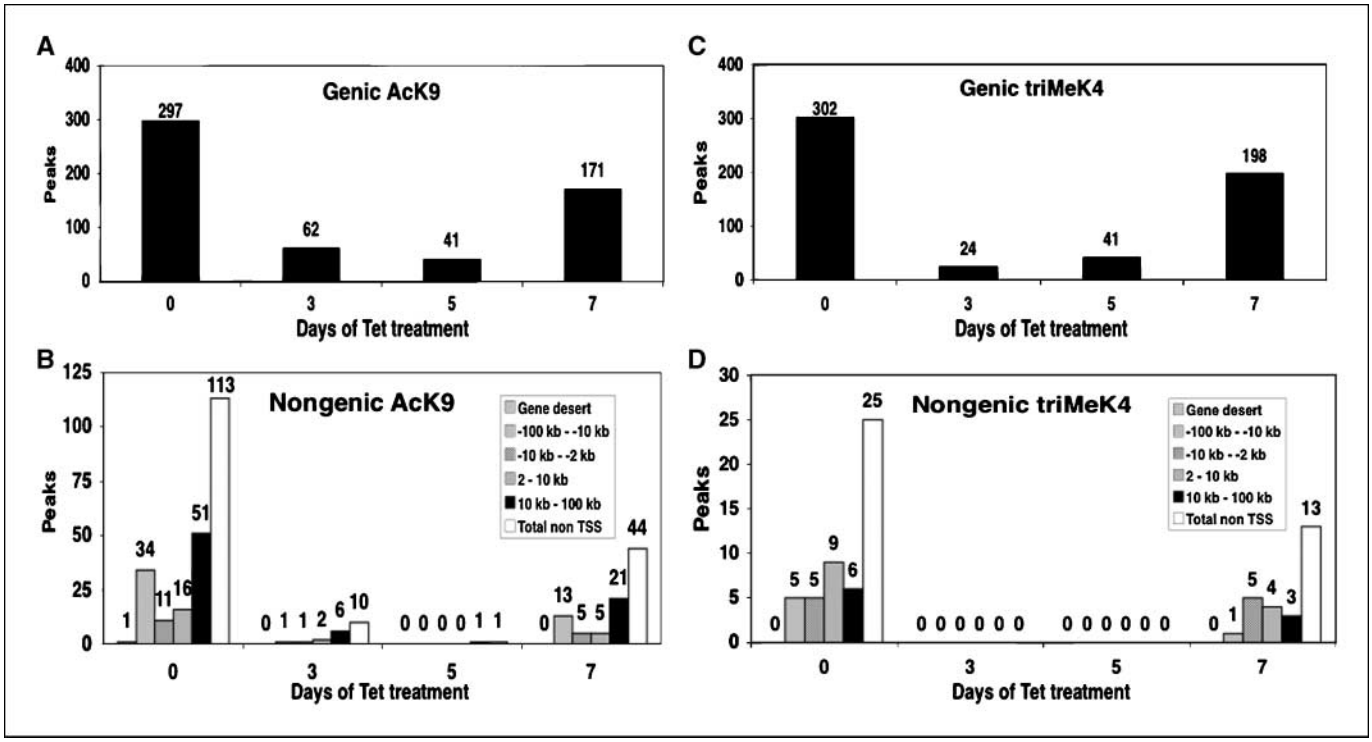


Figure 3. N-Myc regulation of AcK9 and triMeK4 includes both genic and nongenic regions. Bioinformatics analysis of ChIP-chip data subdivided into genic (*A*) and nongenic (*B*) AcK9 domains. Genic (*C*) and nongenic (*D*) triMeK4 domains. Nongenic peaks are presented as subgroups relative to the distance to the TSS. Peaks scored at L1 stringency.

genomic AcK9 and triMeK4 modifications in human neuroblastoma, many of these N-Myc–dependent marks are nonetheless localized at sites without detectable N-Myc direct binding (Fig. 2*A* and *B*). For example, out of 410 AcK9 peaks on the ENCODE array in TET21N cells, 368 (90%) are lost within 5 days of N-Myc depletion, but of those 368, only 116 are N-Myc–bound. Thus, most (252) AcK9 peaks that are N-Myc–dependent are nonetheless not detectably bound by N-Myc and the same applies to triMeK4 peaks. A key question is whether these domains reflect indirect N-Myc activity or if they are false-negatives for N-Myc binding. To test these possibilities, we conducted direct ChIP verification assays on regions, both genic and >10 kb from TSS, that by ChIP-chip were apparently N-Myc unbound, but had N-Myc–dependent AcK9 and triMeK4 (Fig. 2*C*; UBChCh 1–11). Only 1 out of 11 of the tested UBChCh domains (no. 4) clearly showed N-Myc binding by straight ChIP assay, indicating that most regions scored as unbound by N-Myc are not false-negatives and are indeed not bound by N-Myc. The intensity of AcK9 and triMeK4 peaks at unbound regions as well as their degree of dependence on N-Myc are just as robust as at bound regions (Fig. 2*D*). The loss of AcK9 and triMeK4 at these domains does not seem to be due to the effects of Tet alone as Tet has no effect on their levels in parental SHEP cells (Fig. 1*C*). Thus, N-Myc seems to have the ability to regulate AcK9 and triMeK4 at regions it does not bind, indicating that it possesses both direct and indirect mechanisms of regulating chromatin.

The N-Myc–regulated chromatin program is hierarchical and reversible. N-Myc exhibited a total of 146 and 94 binding sites coinciding with AcK9 and triMeK4, respectively, in untreated TET21N cells (Fig. 2*A* and *B*). Only 30 N-Myc–bound AcK9 domains remained at day 5, whereas only 17 N-Myc bound triMeK4 domains remained at day 3, representing approximately 5-fold reductions in both cases. Thus, N-Myc–bound domains of AcK9 and triMeK4 are highly dependent on N-Myc levels for maintenance. However, N-Myc–dependent AcK9 and triMeK4 domains that are not detectably bound by N-Myc are even more sensitive to the loss of N-Myc with approximately 22-fold and 33-fold reductions in AcK9 (day 5) and triMeK4 (day 3) domains, respectively (Fig. 2*A* and *B*). Thus, a hierarchy of N-Myc–regulated chromatin domains seems to exist, with sites not detectably bound by N-Myc being of the lowest affinity and hence most sensitive to reduced N-Myc levels.

AcK9 and triMeK4 are dynamically regulated by N-Myc and importantly their loss is reversed by re-increasing N-Myc levels as evidenced by IB of acid extracts of histones (Fig. 1*C*). At a genomic level, the partial restoration of N-Myc protein levels at day 7 of Tet treatment also has clearly discernable effects on histone modifications detectable by ChIP-chip leading to a strong restoration of AcK9 and triMeK4 sites (Fig. 2*A* and *B*). Remarkably, possibly reflecting an epigenetic memory, ~97% of total day 7 peaks of both AcK9 (209 of 215) and triMeK4 (204 of 211) at day 7 were at sites in which these marks existed previously in day 0 control cells. These data also indicate that the loss of AcK9 and triMeK4 is specifically caused by depletion of N-Myc and is not due to an intermediate irreversible change in cell biology.

N-Myc regulates AcK9 and triMeK4 at remote domains large distances from TSS. A substantial subset, ~40%, of N-Myc binding is at nongenic sites (Fig. 1*B*) from 2 kb to >100 kb from TSS. Although such binding is not typically associated with TF function, AcK9 and triMek4 at these sites is almost completely dependent on N-Myc (Fig. 3), suggesting that N-Myc is mediating these euchromatic modifications despite their distance from the

promoters. Interestingly, the largest subset of nongenic domains is at least 10 to 100 kb from the TSS. These remote from start site domains (RSS), defined here as domains >10 kb away from the nearest TSS, are very rarely bound by most TF. For example, only 8% of E2F1 and only 3% of *Pol*II binding sites are in such regions (28). Nonetheless, AcK9 and triMeK4 at RSS domains are essentially 100% dependent on N-Myc for their maintenance (Fig. 3*B* and *D*), much more sensitive than TSS domain euchromatin (Fig. 3*A* and *C*), suggesting that RSS are bona fide sites of Myc activity.

An example of two representative N-Myc–bound RSS (RSS3–4) is shown in Fig. 4*A*. N-Myc binding at these RSS is specific as it is ablated with Tet treatment. RSS3 and 4 are 100 to 200 kb away from the nearest TSS, yet AcK9 and triMeK4 in RSS3 and 4 are strongly reduced at days 3 to 5 and re-elevated at day 7 (Fig. 4*A, right box*). As a control, we performed straight ChIP assays on seven N-Myc–bound RSS and found all seven confirmed, whereas zero of five ChIPs confirmed enrichment for N-Myc binding or modified histones at randomly selected RSS (RSSr1–5; Fig. 4*B*). The functional consequences of N-Myc's action at RSS remain unknown and we have not found evidence of regulation of the nearest neighbor genes (for example, expression microarray data for genes nearest to RSS3–4; Fig. S3) through an enhancer-like function, but this cannot be ruled out given the unpredictable distribution of the targets of enhancers and the fact that enhancers can be >1 Mb from their targets. The fact that at some RSS, N-Myc regulates both AcK9 and triMeK4 (Fig. 4), whereas at others (Fig. S4) it only regulates AcK9, suggests functional heterogeneity among RSS.

N-Myc bound and regulated genic chromatin domains weakly influence transcription. Parallel gene expression microarray and ChIP-chip data were compared (Table S2). Of the 66 N-Myc bound annotated genes for which a clear result was evident on the array, 63 (96%) had either AcK9 or triMeK4 present with 53 (80%) having both marks. Of the 63 genes with either or both marks, 38 (58%) lost the marks upon N-Myc depletion with 6 of 38 being restored at least 2-fold at day 7. Overall changes in the expression of N-Myc–bound genes were modest, most often in the range of 2-fold or less despite pronounced chromatin modification changes (Fig. 5). Furthermore, the relative degree of loss of AcK9 and triMeK4 (e.g., as determined by peak counting represented by data to the right of the SignalMap histograms) at individual genes with depletion of N-Myc did not clearly correlate with the relative decrease in transcription at the five genes examined (the three genes in Fig. 5 and two additional N-Myc–bound and regulated genes, *DKC1* and *CEP250*). This suggests that even minor decreases in N-Myc–mediated euchromatin are sufficient to drive the approximately 2-fold decrease in transcription that we typically see as a maximum decrease in Myc target genes upon Myc loss.

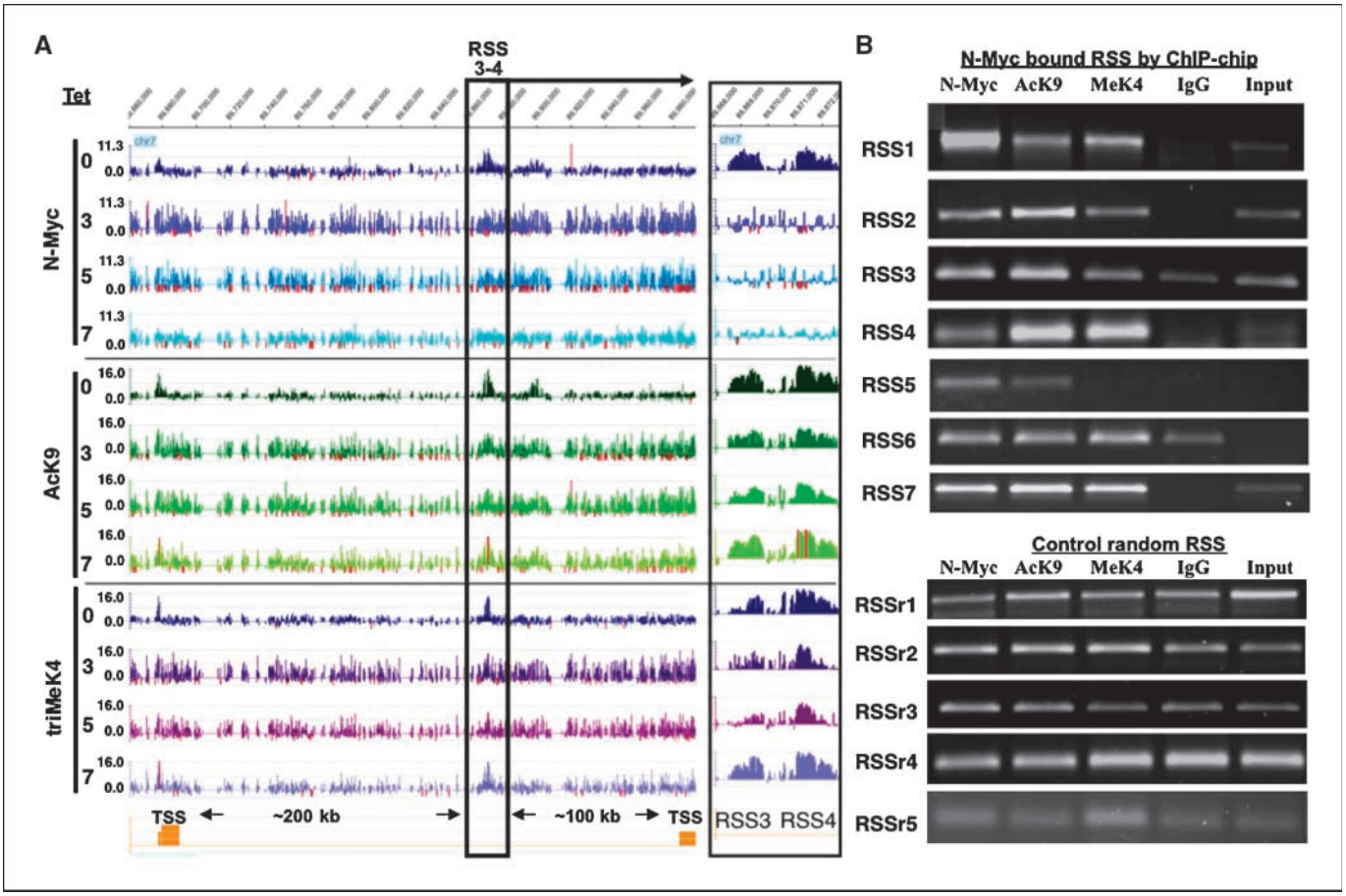


Figure 4. N-Myc–bound and –regulated RSS. *A,* snapshot of SignalMap data remote from transcriptional start site domains (*RSS*) that are bound and regulated by N-Myc. *Inset at right of A,* peaks of RSS3 and RSS4. *B,* ChIP confirmation attempts for seven RSS bound by N-Myc. Enriched N-Myc binding with links to either AcK9 or triMeK4 were observed at seven of seven RSS tested, whereas zero of five random RSS (*RSSr1–5*) exhibited such traits.

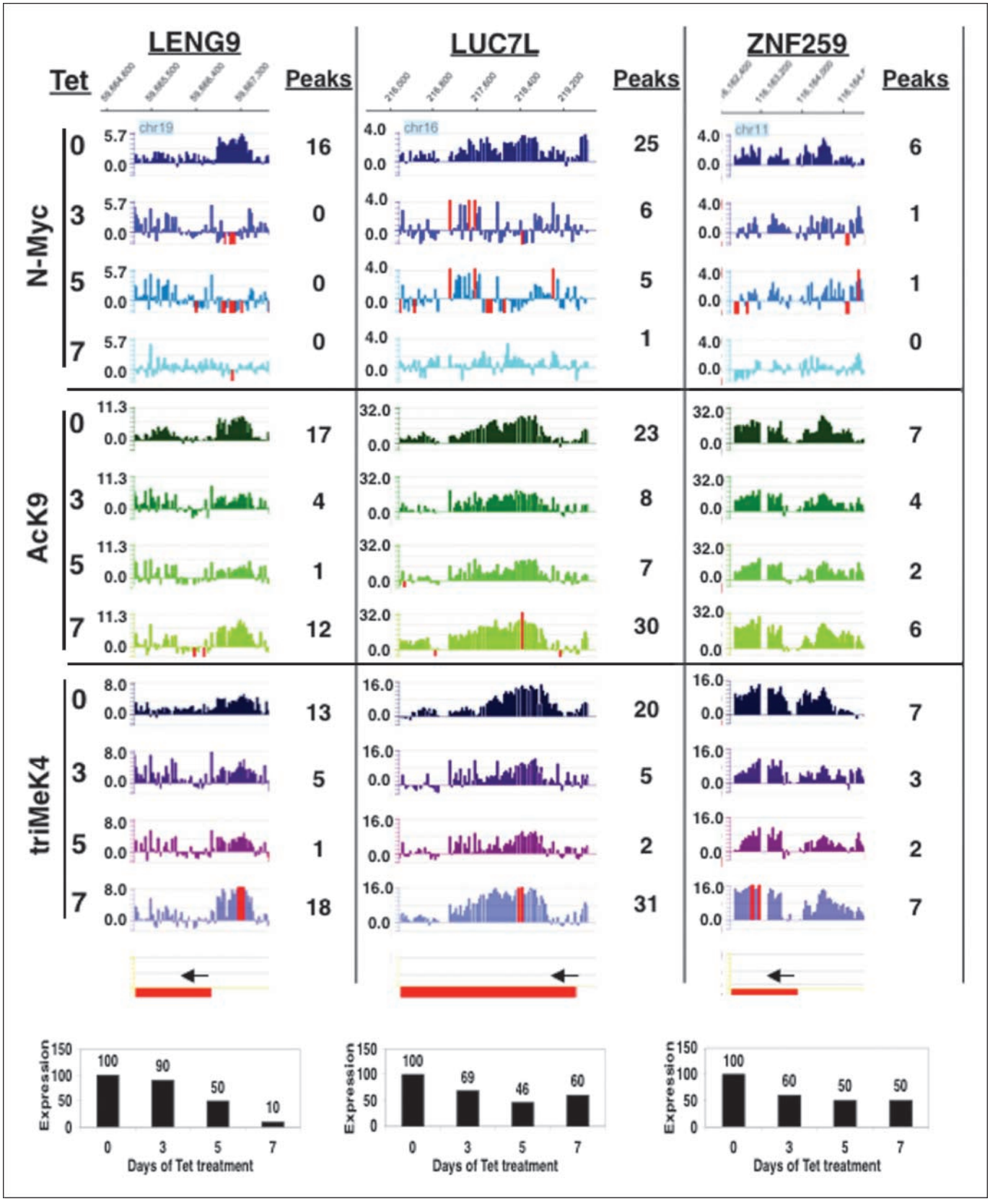


Figure 5. N-Myc is a weak TF even at genes which it directly binds and at which it regulates AcK9 and triMeK4. Snapshots of SignalMap data of three representative genes meeting the following criteria as putative N-Myc targets: N-Myc bound, N-Myc–dependent histone modifications, and reduced levels of gene expression at days 3 to 5. *Data at the bottom for each gene,* mean expression levels from biological replicate expression microarray experiments; *data to the right of each SignalMap histogram,* manually counted peaks per cluster.

Furthermore, Myc-independent transcriptional machinery is likely in place that mediates a certain level of transcription.

Thirty out of the 66 N-Myc–bound genes exhibited day 0 expression at least 10% above day 3 expression levels, but their mean expression at day 0 was just 1.5-fold above day 3 levels for a mean 50% higher expression. Twenty-four N-Myc–bound genes exhibited lower expression at day 0 versus day 3, but only by a mean of 28%. Thus, there is a moderately strong link between our ChIP-chip and gene expression microarray studies. Out of the 66 N-Myc–bound genes, 25 (38%) have been previously identified as Myc target genes (12), suggesting that our ChIP-chip assay efficiently identified targets. At days 3 and 5, 165 and 306 genes were down-regulated 2-fold or more, respectively, suggesting a progressive increase in transcriptional silencing with prolonged depletion of N-Myc (Table S3). The transcriptional effects of loss of N-Myc are not robust, as only 50 and 75 genes are down-regulated 3-fold or more.

Discussion

Myc is linked to widespread histone acetylation (16, 17) as well as overall active histone modifications in tumors and epidermal stem cells (41, 42). Beyond acetylation, Myc is required for the global maintenance of triMeK4 (16), possibly mediated by interaction with the histone demethylase LID, which targets triMeK4 for demethylation (32). Myc targets in ES cells also have a strong, nearly 100% association with triMeK4 (18). Here, for the first time, we use functional genomics to globally map Myc's chromatin function, finding a widespread role for Myc in the human genome including both genic and intergenic regions.

Myc requires a preexisting active chromatin state to bind and in turn influence chromatin itself (17). By combining a loss of function model with functional genomics, we have focused on defining Myc function after it binds. It is of interest, however, that the requirement of Myc for preexisting domains of AcK9 and triMeK4 to bind chromatin so clearly matches with Myc's function to regulate AcK9 and triMeK4 after binding. This suggests possible positive autoregulation of its own chromatin function by Myc. Furthermore, the fact that essentially 100% of AcK9 and triMeK4 marks at day 7 in our studies, when N-Myc is restored, exist at the same locations where they existed at day 0 in the first place, suggests that a specific epigenetic memory exists for Myc binding and function, most likely consisting of low levels of residual AcK9, triMeK4, or some combination of the two. Together, our data and the requirement of Myc for preexisting AcK9 and triMeK4 suggests that N-Myc predominantly maintains or enhances euchromatin and less often establishes it *de novo*.

Open chromatin and open questions. Although there is growing evidence that Myc has a much more global role in the regulation of transcription and chromatin than previously anticipated, this is the first functional genomics study that examines Myc chromatin activity using a loss of function model and that includes intergenic regions distant from classic promoters. We find strong evidence (mainly functional genomics, but also including immunostaining in Fig. S5) that Myc is required to maintain euchromatin in a widespread manner, including at intergenic sites. Although the functional meaning of such activity remains to be determined, two main possibilities exist. First, Myc maintains widespread intergenic euchromatin together with genic euchromatin as part of a global role in sustaining active overall nuclear chromatin. Second, Myc mediates euchromatin at remote sites as part of a previously uncharacterized enhancer function to regulate genes at a distance. At this point, data does not clearly support one model over the other. Nonetheless, it is intriguing that intergenic binding sites for N-Myc are not enriched for E-boxes. Although E-box–independent binding has been reported and may be fairly widespread (17), here, we provide the first evidence that such binding may be of particular importance for Myc intergenic function.

In the model system we have used in this study, the increase in N-Myc at day 7 gives us a unique tool to study the effects of reintroduction of N-Myc and the partial reversal of loss of euchromatic marks at that time suggests that N-Myc regulates a dynamic euchromatic program. One puzzle is why we do not observe an increase in N-Myc binding at that later time point coincident with the chromatin rescue using ChIP-chip. The simplest explanation is that N-Myc is acting at these domains to restore active chromatin, but that its binding is of an affinity too low to detect by ChIP-chip or by direct ChIP (Fig. S7). It is also possible, going along with this notion, that the loss of N-Myc sensitizes chromatin to re-elevation of N-Myc such that a relatively weak amount of N-Myc activity has a more potent effect. A second explanation is that a significant part of the rescue at day 7 is indirect, perhaps because N-Myc induces a target gene such as *GCN5*.

Another surprising observation in our studies was that a large fraction of euchromatic marks regulated by N-Myc were not at locations of detectable N-Myc binding. Although it is formally possible that N-Myc is binding these regions in a manner undetectable by ChIP-chip, we believe it may be more likely that one of three other models or some combination of the three are at work: (*a*) Myc regulates the expression of histone-modifying enzymes (e.g., GCN5; Fig. S6) which are themselves Myc target genes and are responsible for the Myc-dependent chromatin effects at unbound regions; (*b*) Myc regulates the activity of histone-modifying enzymes (e.g., LID/RPB2); and (*c*) Myc regulates chromatin at a distance such that Myc binding at one location can influence chromatin at another through higher order chromatin structure. The fact that partially restored N-Myc at day 7 seems to rescue a substantial fraction of AcK9 and triMeK4 marks that were lost upon its depletion but that N-Myc binding is not detectable at that time similarly suggests either an indirect function for Myc or a type of binding that is not detectable by ChIP-chip but is nonetheless functional.

Although Myc is most well known for its role in cancer, normal Myc functions in stem cells are likely of great importance. Myc's role in the formation of iPS cells is intriguing (43), but the mechanisms by which it enhances iPS cell formation remain unknown. One theory is that Myc contributes to their induced self-renewal and pluripotency through global chromatin reprogramming (13), which may, in essence, set the overall chromatin table for the subsequent activity of other stem cell–related TFs, even if it does not directly interact with those factors (18), a notion consistent with our findings in this study. However, other mechanisms are possible. The function of Myc in iPS cells is likely quite similar to its role in ESC. A specific global hyperactive chromatin state in ESC encompassing expansive intergenic regions, apparently key to the maintenance of an ESC state (44), could be regulated by Myc in ESC and locked in place during tumorigenesis such as neuroblastoma genesis. Ongoing functional genomics analyses of Myc and histone modifications in hESC and iPS cells should resolve many of these open questions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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