Association with Aurora-A Controls N-MYC-Dependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle

Graphical Abstract

Highlights

- N-MYC forms complexes with TFIIIC, RAD21, and TOP2A
- TFIIIC recruits RAD21 and is required for N-MYC-dependent pause release of Pol II
- Aurora-A displaces TFIIIC, TOP2A, and RAD21 from N-MYC during S phase
- Aurora-A inhibits pause release of Pol II during S phase

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In Brief

Büchel et al. demonstrate that N-MYC forms complexes with TFIIIC, TOP2A, and RAD21. Aurora-A competes with TFIIIC and RAD21 for binding to N-MYC, and Aurora-A displaces the three proteins from N-MYC during S phase. As a consequence, N-MYC-dependent pause release is inhibited during S phase, preventing activation of the ATR checkpoint kinase.

Data and Software Availability

GSE78957
Association with Aurora-A Controls N-MYCDependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle

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SUMMARY

MYC proteins bind globally to active promoters and promote transcriptional elongation by RNA polymerase II (Pol II). To identify effector proteins that mediate this function, we performed mass spectrometry on N-MYC complexes in neuroblastoma cells. The analysis shows that N-MYC forms complexes with TFIIIC, TOP2A, and RAD21, a subunit of cohesin. N-MYC and TFIIIC bind to overlapping sites in thousands of Pol II promoters and intergenic regions. TFIIIC promotes association of RAD21 with N-MYC target sites and is required for N-MYC-dependent promoter escape and pause release of Pol II. Aurora-A competes with binding of TFIIIC and RAD21 to N-MYC in vitro and antagonizes association of TOP2A, TFIIIC, and RAD21 with N-MYC during S phase, blocking N-MYC-dependent release of Pol II from the promoter. Inhibition of Aurora-A in S phase restores RAD21 and TFIIIC binding to chromatin and partially restores N-MYC-dependent transcriptional elongation. We propose that complex formation with Aurora-A controls N-MYC function during the cell cycle.

INTRODUCTION

The MYC family of transcription factors (MYC, N-MYC, and L-MYC) has been causally implicated in the development of multiple human tumors (Dang, 2012). MYC proteins bind to virtually all promoters with an open chromatin structure as well as to thousands of enhancers (Walz et al., 2014; Sabó et al., 2014; Lin et al., 2012; Nie et al., 2012). They stimulate transcription of large groups of genes encoding, among others, proteins involved in protein translation, cell cycle progression, and intermediary metabolism and repression of genes encoding cell cycle inhibitory proteins as well as proteins involved in cell adhesion and differentiation (Dang, 2012). In some settings, MYC proteins act as “general amplifiers” that enhance transcription of all actively transcribed protein-coding genes (Nie et al., 2012; Lin et al., 2012).

During early transcription, RNA polymerase II (Pol II) goes through a series of intermediate states that begin with the formation of a closed promoter complex and end with the release of Pol II from a promoter-proximal pause position into productive elongation (Michel and Cramer, 2013). To enhance transcription, MYC proteins promote both recruitment of Pol II to the promoter and its subsequent release into elongation (Rahl et al., 2010; Jaenicke et al., 2016; Walz et al., 2014). Escape from the promoter and pause release of Pol II are controlled by sequential phosphorylation of Ser5 and Ser2 in the C-terminal domain of Pol II and activation of MYC promotes phosphorylation at both sites (Jonkers and Lis, 2015; Rahl et al., 2010; Walz et al., 2014; Cowling and Cole, 2007). Known protein-protein interactions of MYC include the association with MAX, which is required for binding to DNA (Blackwell et al., 1993), the NuA4 histone acetylase complex (McMahon et al., 1998), the p400 and BPTF chromatin-remodeling complexes (Richart et al., 2016; Fuchs et al., 2001), and the PAF1C elongation complex (Jaenicke et al., 2016). How MYC engages its different
cofactors to promote elongation and how this process is regulated is largely unknown.

Deregulated expression of N-MYC is characteristic for subentities of pediatric neuroblastoma and medulloblastoma as well as for adult neuroendocrine and some hematopoietic malignancies (Beltran, 2014). Like all MYC proteins, N-MYC is rapidly turned over via the ubiquitin/proteasome system (Sjostrom et al., 2005). The SCF<sup>FBXW7</sup> ubiquitin ligase recognizes a phosphodegron that is part of a conserved domain called MYCBox (Welcker et al., 2004). Replacing two phosphoresidues, T58 and S62, with alanine stabilizes N-MYC (Otto et al., 2009). S62 is phosphorylated by cyclin-dependent kinases and primes phosphorylation of T58 by GSK3, resulting in cell cycle- and growth factor-dependent proteasomal turnover of N-MYC (Chesler et al., 2006; Sjostrom et al., 2005).

In multiple tumor entities, N-MYC is stabilized by complex formation with Aurora-A (Otto et al., 2009; Dardenne et al., 2016). Aurora-A is a serine/threonine kinase that is best known for its role in centrosome function and mitosis (Marumoto et al., 2005). Aurora-A binds to N-MYC directly via two domains that flank the SCF<sup>FBXW7</sup> phosphodegron and antagonizes degradation of N-MYC by SCF<sup>FBXW7</sup> (Otto et al., 2009; Richards et al., 2016). Although stabilization of N-MYC does not require Aurora-A catalytic activity, Aurora-A inhibitors that distort the kinase domain dissociate the N-MYC/Aurora-A complex and destabilize N-MYC (Richards et al., 2016). These inhibitors show therapeutic efficacy in N-MYC-driven tumor models and are being tested in human patients (DuBois et al., 2016). We have now analyzed protein complexes of N-MYC to understand how N-MYC controls Pol II function and how association with Aurora-A impacts N-MYC function.

RESULTS

Complexes of N-MYC with TFIIIC, TOP2A, and RAD21

To identify protein complexes of N-MYC in neuroblastoma cells, we used SH-EP cells, which do not express endogenous N-MYC. Using retroviral infection, we stably expressed HA-tagged wild-type (WT) N-MYC (N-MYC<sub>wt</sub>) or N-MYCT58AS62A (N-MYC<sub>mut</sub>) (Figure 1A). Immunoblot showed that two N-MYC subunits in infected cells were comparable with those found in a MYCN-amplified neuroblastoma cell line, IMR-32 (Figure 1A). We then performed mass spectrometry of complexes recovered after immunoprecipitation with α-hemagglutinin (α-HA) antibodies and elution with a HA peptide using label-free quantification (Figure 1B; Table S1). The analysis confirmed multiple well-validated interactions of N-MYC or MYC proteins, including interactions with TRRAP, p400, BPTF, MAX, Aurora-A, and CDC73 (Figure S1A). In addition, the analysis identified interactions with 5 of 6 subunits of the TFIIIC complex and topoisomerases IIA and IIB (TOP2A and TOP2B). TFIIIC is both a general transcription factor of RNA polymerase III and an architectural protein that is localized at the border of topological domains (Crepaldi et al., 2013; Van Bortle et al., 2014). TOP2A has been implicated in relieving transcription-induced supercoiling at highly active promoters (Kouzine et al., 2013). We speculated, therefore, that these interactions may identify effector functions of N-MYC.

Immunoprecipitations using α-HA antibodies confirmed that TFIIIC and TOP2A were present in immunoprecipitates from cells expressing N-MYC but not in control immunoprecipitates from cells not expressing HA-tagged N-MYC (Figure S1B). Parallel mass spectrometry demonstrated the presence of 5 of 6 subunits of TFIIIC and TOP2A in α-N-MYC immunoprecipitates but not in control immunoprecipitates from MYCN-amplified neuroblastoma cells (Figure S1C). Multiple subunits of TFIIIC and TOP2A were also detected in α-HA precipitates from U2OS cells expressing HA-tagged MYC but not control immunoprecipitates, arguing that MYC, like N-MYC, associates with these proteins (Figure S1D); this is consistent with a previous mass spectrometric analysis (Koch et al., 2007). Using an antibody that recognizes TFIIIC5, we confirmed that TFIIIC associates with endogenous N-MYC and, albeit more weakly, with TOP2A as well as TOP2B in MYCN-amplified neuroblastoma cells (Figure 1C; Figure S1E). Notably, interactions of N-MYC with TFIIIC5 and TOP2A were also detected in the presence of ethidium bromide (EtBr), which disrupts DNA-dependent interactions (Figure 1C). This observation precludes the possibility that the interactions are indirectly mediated by DNA.

To identify domains of N-MYC that interact with TFIIIC and TOP2A, we performed pull-down assays from cell lysates incubated with recombinant FLAG-tagged peptides covering fragments of the N-MYC N terminus (Figure 1D). These assays showed that residues 1–137 are sufficient for binding to TFIIIC and suggested that two regions of N-MYC that flank MYCBoxI, but not MYCBoxII itself (peptides 46–89), mediate binding to TFIIIC. Controls established that an equimolar amount of an unrelated FLAG-tagged protein (heterogeneous nuclear ribonucleoprotein [hnRNP], amino acids 330–463) does not bind TFIIIC5 (E.L., unpublished data). In parallel assays, we were unable to demonstrate that residues 1–137 of N-MYC are sufficient for binding to TOP2A (E.L., unpublished data). Previous work has demonstrated that TFIIIC co-localizes and associates with cohesin and condensin complexes in different organisms (Van Bortle et al., 2014; D’Ambrosio et al., 2008), prompting us to search for the presence of these complexes in N-MYC immunoprecipitates. Indeed, peptides of the kleisin component of the cohesin complex, RAD21, were found in the mass spectrometry analysis (Table S1), and we confirmed that both N-MYC expressed in SH-EP cells and endogenous N-MYC in MYCN-amplified IMR-5 cells associates with RAD21 (Figure 1E; Figure S1B). Mapping of the interaction to the N-MYC N terminus showed the same binding pattern as TFIIIC, suggesting that they co-exist with N-MYC in the same complex (Figure 1D).

Like TFIIIC and RAD21, Aurora-A interacts with N-MYC via two domains flanking MYCBox (Richards et al., 2016), raising the possibility that Aurora-A competes with TFIIIC or RAD21 for binding to N-MYC. Indeed, Aurora-A competed with both TFIIIC and RAD21 for binding to the N-MYC N terminus (1–137) in pull-down assays using FLAG-tagged peptides, whereas an equimolar amount of an unrelated protein, glutathione-S-transferase, did not compete (Figure 1F). Immunoprecipitations from MYCN-amplified neuroblastoma cells showed that TFIIIC, RAD21, and Aurora-A were present in α-N-MYC immunoprecipitates (Figure 1E). In parallel assays, both N-MYC and, weakly, TFIIIC, but not RAD21, were detectable in α-Aurora-A
immunoprecipitates (Figure 1E). We concluded that Aurora-A and RAD21 form alternate complexes with N-MYC in cells, whereas Aurora-A can remain associated with N-MYC and TFIIIC via interactions that are outside of residues N-MYC 1–137 in vivo. To identify these domains in N-MYC, we used overlapping peptide libraries in microarray format of N-MYC and probed them with recombinant Aurora-A. Consistent with these and previously published data (Richards et al., 2016), peptides spanning amino acids 17–43 of N-MYC (9–43 in MYC) had the highest affinity for Aurora-A. The arrays also revealed an additional binding site with lower binding affinity for Aurora-A in the C terminus of N-MYC, which spans amino acids 313–339 (overlapping MYCBoxIV) and is conserved in MYC (amino acids 306–331) (Figure S1F).

The interaction of Aurora-A with N-MYC is impaired by mutations of T58 and S62 in N-MYC to alanine (Otto et al., 2009), and immunoprecipitations from infected SH-EP cells confirmed this observation (Figure S1B). Comparison of precipitates recovered from cells expressing N-MYCwt with those from cells expressing N-MYCmut suggested that phosphorylation of MYCBoxI may...
affect the interaction with TFIIIC (Figure 1B), but immunoprecipitations from SH-EP cells did not reveal significant differences in the interactions of N-MYC wt and N-MYCmut with RAD21, TFIIIC5, and TOP2A (Figure S1B).

**Joint N-MYC/TFIIIC Chromatin Binding Sites**

TFIIIC binds to promoters transcribed by RNA polymerase III and is an architectural protein complex that binds to thousands of ETC (extra TFIIIC) sites throughout the genome that are independent of RNA polymerase III (Moqtaderi et al., 2010; Oler et al., 2010). To understand whether N-MYC and TFIIIC bind to overlapping sites on chromatin, we performed chromatin immunoprecipitation (ChIP) coupled with high-throughput sequencing (ChIP sequencing) using antibodies directed against N-MYC and TFIIIC5. Inspection of individual genes revealed the presence of both proteins in close vicinity to each other at multiple transcription start sites (Figure 2A; Figure S2A). Global analyses identified a total of 2,053 sites with overlapping peaks, and statistical analyses showed that this overlap is highly significant (Figure 2B) \( (p < 10^{-6}) \), calculated using a permutation test with 1.0 \( \times 10^6 \) iterations. The median distance between peak summits at all joint sites was 39 bp (Figure S2B). Of these sites, 151 were found at tRNA genes; this is expected because MYC proteins bind to tRNA genes (Gomez-Roman et al., 2003). In addition, overlapping N-MYC/TFIIIC sites were found at 1,165 promoters transcribed by Pol II and at 737 intergenic sites. At core promoters transcribed by Pol II, N-MYC binding peaked at transcription start sites, and TFIIIC binding peaked with a slightly larger median distance of 80 bp of N-MYC binding sites, suggesting that joint sites may have a defined orientation (Figure S2C).

MYC proteins bind to E-box sequences (CAC(A/G)TG) as part of a heterodimeric complex with MAX (Blackwell et al., 1993). Consistently, a de novo motif search identified E-boxes as a
predominant motif enriched in N-MYC binding sites in Pol II promoters and at N-MYC/TFIIIC joint intergenic sites (Figure 2C; Figure S2D). TFIIIC promotes binding to a sequence termed A-box at tRNA promoters that are not present at ETC sites (Figure 2C; Figure S2D; Moqtaderi et al., 2010). In addition, TFIIIC binds to a sequence termed B-box that is present in tRNA promoters and in ETC sites and the de novo motif search confirmed these observations (Figure 2C; Figure S2D; Moqtaderi et al., 2010). B-boxes were also found in overlapping N-MYC/TFIIIC sites in core promoters.

In addition to TFIIIC, ETC sites are also bound by the CTCF transcription factor (Moqtaderi et al., 2010; Oler et al., 2010; Carrière et al., 2012; Vietri Rudan and Hadjur, 2015). Indeed, a de novo motif search analysis identified a centrally enriched consensus motif for CTCF at joint N-MYC/TFIIIC binding sites (Figures 2C and 2D; Figures S2D–S2F). ChIP sequencing confirmed the presence of CTCF at 936 of 2,053 joint N-MYC/TFIIIC binding sites and showed a much lower occupancy at N-MYC sites that do not bind TFIIIC (Figures 2E and 2F; Figure S2G). We concluded that N-MYC is present at previously characterized TFIIIC binding sites both in RNA polymerase III promoters and at ETC sites.

N-MYC and TFIIIC Promote Chromatin Association of RAD21 at Joint Binding Sites

CTCF binding sites define contact points for RAD21/cohesin-mediated chromosomal interactions (Ghirlando and Felsenfeld, 2016). Consistent with this notion, ChIP sequencing showed that RAD21 was present at virtually all (22,642 of 23,479) CTCF-bound sites and at 1,328 of 2,053 joint N-MYC/TFIIIC sites (Figures 2E and 3A). RAD21 occupancy was much lower at N-MYC sites that do not bind TFIIIC (Figure 2F). We also observed that endogenous TFIIIC5 robustly co-immunoprecipitated endogenous RAD21 (Figure 3B). Because RAD21/cohesin complexes do not bind DNA directly, these observations raised the question of whether TFIIIC or N-MYC affects chromatin association of each other and of RAD21. We therefore performed ChIP experiments in cells stably expressing doxycycline-inducible short hairpin RNAs (shRNAs) targeting either TFIIIC or N-MYC. Depletion of TFIIIC5 had little effect on steady-state levels of N-MYC, RAD21, Aurora-A, and the DNA binding subunit TFIIIC2 but led to a small reduction in TFIIIC1 levels (Figure 3C; Figure S3A; Shen et al., 1996). As expected, we observed reduced binding of TFIIIC5 at multiple N-MYC-bound loci (Figure 3D). Depletion of TFIIIC5 had no effect on binding of N-MYC but reduced binding of RAD21 at almost all tested joint binding sites, demonstrating that TFIIIC5 promotes binding of RAD21 (Figure 3D). In contrast, depletion of TFIIIC5 had no effect on RAD21 association with sites that were not bound by TFIIIC and N-MYC (Figure S3B). Notably, shRNA-mediated depletion of TFIIIC5 strongly reduced TFIIIC5 occupancy at sites transcribed by Pol II, but TFIIIC5 binding to tRNA sites was more stable (Figure S3C). Although RAD21 is not detected at tRNA genes (Oler et al., 2010; Moqtaderi et al., 2010), the stable binding of TFIIIC5 to tRNA encoding genes precluded an unequivocal determination of whether TFIIIC5 promotes binding of N-MYC to tRNA genes. We also depleted TFIIIC2 and found that this has no effect on either N-MYC or RAD21 binding to joint sites, arguing that not all subunits of TFIIIC are limiting for chromatin association of RAD21 (Figures S3A and S3D). Depletion of N-MYC using a doxycycline-inducible shRNA reduced binding of N-MYC to most joint sites and significantly reduced binding of both TFIIIC5 and of RAD21 to some sites, although the extent of reduction was more variable than observed after TFIIIC5 depletion (Figures 3E and 3F). In contrast, depletion of N-MYC did not decrease RAD21 association with sites that were not bound by TFIIIC and N-MYC (Figure S3E). Taken together, the results suggest a hierarchy of chromatin binding in which N-MYC contributes to recruitment of both TFIIIC5 and RAD21 and TFIIIC5 is required for association of RAD21 with joint binding sites.

Transcriptional Regulation by N-MYC and TFIIIC/RAD21

To determine whether TFIIIC5 and RAD21 are required for the transcription of N-MYC target genes, we depleted either protein in IMR-5 cells using specific siRNAs. Control immunoblots are shown in Figure 4A. RNA sequencing of two independent samples for each protein showed a significant overlap of genes regulated in response to depletion of either protein (Figure 4B). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) showed that multiple well-characterized sets of MYC-activated target genes were significantly downregulated upon depletion of TFIIIC5 or RAD21; in contrast, only few gene sets were significantly upregulated upon depletion of TFIIIC5 or RAD21 (Figures 4C and 4D). GSEA showed that the most consistently downregulated gene sets encoded proteins involved in cell cycle progression, DNA replication, nucleotide metabolism, and telomere biology (Figure 4D). Comparison with expression profiles obtained after shRNA-mediated depletion of N-MYC in neuroblastoma cells (Valentijn et al., 2012) confirmed that expression of these genes is sensitive to depletion of N-MYC (Figures 4C and 4E). Furthermore, expression of gene sets that are sensitive to depletion of TFIIIC, RAD21, and N-MYC strongly correlated with MYCN amplification and advanced tumor stage in neuroblastoma, both of which are characteristic features of aggressive tumors with a poor prognosis (Figure 4F; Molenaar et al., 2012). We concluded that TFIIIC and RAD21 are required for expression of a subset of N-MYC target genes that are characteristic for MYCN-amplified neuroblastoma tumors. Consistent with these effects on gene expression, shRNA-mediated depletion of TFIIIC5 protein suppressed the growth of neuroblastoma cells (Figure S4A), although it had little effect on the expression of two RNA genes we tested (Figure S4B). Notably, growth suppression by depletion of TFIIIC5 was independent of MYCN amplification (Figure S4A), arguing that TFIIIC5 also has essential functions in non MYCN-amplified cells. Most likely, the residual growth of IMR-5 cells after shRNA-mediated depletion of TFIIIC5 was due to incomplete removal of TFIIIC5 because multiple sgRNAs targeting TFIIIC5 eliminated cell growth (Figure S4C). This is consistent with the demonstration that subunits 1–5 of TFIIIC, RAD21, and TOP2A have been identified as essential proteins in genome-wide CRISPR-based loss-of-function screens (Hart et al., 2015). Depletion of TFIIIC5 caused little apoptosis and, like depletion of N-MYC (Otto et al., 2009), delayed progression through all phases of the
Figure 3. Assembly of N-MYC/TFIIIC and RAD21 Complexes on Chromatin
(A) Venn diagram documenting genome-wide overlap of N-MYC/TFIIIC5 joint binding sites with RAD21 binding sites. The p value was calculated using a permutation test with 100,000 iterations.

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cell cycle; hence, fluorescence-activated cell sorting (FACS) analyses did not detect a major shift in cell cycle distribution (Figure S4D).

(B) Immunoblots of α-TFIIIC5 immunoprecipitates from IMR-5 cells. The input corresponds to 1% of the amount used for the precipitation. Non-specific IgG was used for control immunoprecipitates. Where indicated, CD532 (1 μM) was added to cells 4 hr prior to immunoprecipitation (n = 3).

(C) Immunoblot showing levels of the indicated proteins in response to depletion of TFIIIC5. IMR-5 cells expressing an inducible shRNA directed against TFIIIC5 were treated with doxycycline (Dox) for 48 hr or with ethanol (EtOH) as a control (n = 3).

(D) ChIP experiments documenting binding of TFIIIC5, N-MYC, and RAD21 to the indicated loci upon depletion of TFIIIC5. Error bars show SD of technical triplicates from one experiment (n = 2).

(E) Immunoblot showing levels of the indicated proteins in response to depletion of N-MYC. IMR-5 cells expressing a Dox-inducible shRNA against N-MYC were treated with Dox for 48 hr or with EtOH as a control (n = 3).

(F) ChIP experiments documenting binding of TFIIIC5, N-MYC, and RAD21 to the indicated loci upon depletion of N-MYC. Error bars show SD of technical triplicates from one experiment (n = 2).

See also Figure S3. n indicates the number of independent biological replicas for each experiment.

Cell Cycle-Dependent Regulation of N-MYC Binding and Chromatin Association

Aurora-A has been implicated in cell cycle progression, prompting us to analyze complex formation between N-MYC and Aurora-A throughout the cell cycle in a semiquantitative manner using proximity ligation assays (PLAs) (Söderberg et al., 2006). In PLAs, a rolling circle amplification using oligonucleotides bound to secondary antibodies generates a signal that appears as a fluorescent dot when two antigens are in proximity of each other. Controls using a N-MYC

See also Figure S4. n indicates the number of independent biological replicas for each experiment.

Cell Cycle-Dependent Regulation of N-MYC, TFIIIC, and RAD21

(A) Immunoblots documenting levels of the indicated proteins 48 hr after transfection of specific siRNAs. Duplicate samples are shown, both of which were used for RNA sequencing. All lanes are from the same exposure of a single immunoblot. (B) Venn diagrams documenting the overlap of upregulated (top) and downregulated (bottom) genes after depletion of TFIIIC5 or RAD21 in IMR-5 neuroblastoma cells. The p values were calculated using a Monte Carlo simulation with 100,000 permutations and all expressed genes (n = 17,450) as the basis.

(C) Correlation of gene sets that change in expression upon depletion of N-MYC with the aggregate of changes in response to siRNA-mediated depletion of RAD21 and TFIIIC5. Each dot reflects a gene set. A light gray color indicates that the expression change of a gene set was not statistically significant. Published sets of MYC target genes are colored.

(D) Examples of gene sets that are downregulated in response to depletion of both RAD21 and TFIIIC5. NES is the normalized enrichment score, indicating direction and extent of regulation.

(E) Boxplots documenting changes in expression of selected gene sets upon depletion of N-MYC using a Dox-inducible shRNA in IMR-32 neuroblastoma cells.

(F) Heatmap illustrating stage-specific expression of N-MYC/TFIIIC/RAD21-regulated genes sets in human neuroblastoma cells. The black bars in the first row indicate MYCN amplification status. See also Figure S4. n indicates the number of independent biological replicas for each experiment.
Figure 5. Regulation of N-MYC Transcription Complexes during the Cell Cycle

(A) Representative pictures from proximity ligation assays (PLAs) documenting complex formation between N-MYC and Aurora-A in IMR-5 cells after release from a double thymidine block. Non-synchronized cells are shown as a control (Ctr). Nuclei were stained using Hoechst. Red dots show PLA signals resulting from N-MYC/Aurora-A interactions (n = 3).

(B) Counts of PLA signal

(C) DNA content

(D) N-MYC +

(E) N-MYC + Aurora-A N-MYC + RAD21 N-MYC + TOP2A N-MYC + TFIIIC5

(F) Input [%]

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antibody in SH-EP cells that do not express N-MYC (Figure S5A) or using no primary antibody (Figure S5B) established that the signals observed in these assays are specific. We used either a release from a double thymidine block (Figure S5C) or re-stimulation of serum-starved cells (Figure S5D) to synchronize cells. In both experimental settings, Aurora-A predominantly associated with N-MYC during the S phase of the cell cycle, and complex formation was regulated independently of changes in overall N-MYC and Aurora-A protein levels (Figures S5A, S5B, and S5E; Figures S5E and S5F). We next tested whether association of N-MYC with cofactors changes throughout the cell cycle. Neither the association of N-MYC with p400 and TRRAP, which is mediated by MYCBoxII (amino acids 128–143), nor the interactions with the PAF1 components CTR9 and CDC73, for which the interaction domains are unknown, varied throughout the cell cycle (Figure S5G). In contrast, association of RAD21 with N-MYC was essentially absent during S phase (Figures S5C–S5E). Similarly, associations of N-MYC with TOP2A and TFIIIC5 changed during cell cycle progression and were significantly lower in S phase (Figures S5C–S5E; Figure S5H). ChIP experiments from cells harvested in G1 and S phase showed that association of N-MYC with joint binding sites was essentially equal in both cell cycle phases, whereas chromatin association of RAD21 was strongly reduced in S relative to G1 phase (Figure 5F). Chromatin association of TFIIIC was also reduced in S phase, but the extent of decrease was more variable between different gene loci. We concluded that complex formation of N-MYC with RAD21, TFIIIC, and TOP2A and chromatin binding of RAD21 and TFIIIC are reduced during S phase.

**Aurora-A Antagonizes N-MYC/RAD21 Complex Formation in S Phase**

We showed previously that association with N-MYC activates the catalytic activity of Aurora-A (Richards et al., 2016), and large-scale proteomic analyses show that multiple proteins associated with N-MYC, including TFIIIC2 and TOP2A, are phosphorylated in an Aurora-A–dependent manner in vivo (Kettenbach et al., 2011). These findings, and the observation that Aurora-A competes with association of RAD21 with N-MYC, led us to hypothesize that Aurora-A may antagonize complex formation of N-MYC with RAD21, TOP2A, and, potentially, TFIIIC during S phase. To test this, we used three structurally well-characterized Aurora-A inhibitors to acutely antagonize Aurora-A (Figure 6A; Richards et al., 2016). Of the inhibitors used, MK-5108 is a purely catalytic Aurora-A inhibitor that does not alter the conformation of Aurora-A and does not affect complex formation with N-MYC. MLN8237 inhibits both the catalytic activity of Aurora-A and moderately distorts the N-MYC/Aurora-A complex, enhancing degradation of N-MYC by FBXW7 in mitosis. CD532 strongly distorts the Aurora-A structure, dissociates the N-MYC/Aurora-A complex, and induces subsequent degradation of Aurora-A and N-MYC (Richards et al., 2016). We confirmed that CD532 causes a proteasome-dependent decrease in N-MYC and Aurora-A levels (Figures S6A and S6B). In contrast, CD532 had no effect on complex formation of TFIIIC5 with RAD21 (Figure 3B).

PLA assays from cells released for 4 hr from a double thymidine block into S phase showed that MK-5108 and MLN8237 enhanced interactions of N-MYC with TFIIIC5, TOP2A, and, albeit more weakly, RAD21 (Figures 6B and 6C). In these assays, MK-5108 had the strongest effect. CD532 and MLN8237 had smaller and more variable effects in PLAs with TOP2A, RAD21, and TFIIIC5, most likely because the observed signals reflect a combination of the decrease in N-MYC levels and an increase in interaction (Figures 6A–6C). The data argue that Aurora-A–dependent phosphorylation antagonizes interactions of N-MYC with all three proteins. ChIP and ChIP sequencing showed that chromatin association of N-MYC decreased in response to CD532, consistent with the effects on protein levels (Figures 6D and 6E; Figure S6C). In contrast, CD532 globally increased TFIIIC binding to chromatin (Figure 6D; Figure S6D), and the number of joint N-MYC/TFIIIC binding sites increased to 7,994 in the presence of CD532 (Figure S6E). Note that N-MYC binding to chromatin remains detectable at virtually all sites after 4 hr of CD532 treatment. ChIP assays confirmed that CD532 enhanced stable chromatin binding of TFIIIC and RAD21 at joint binding sites, whereas the effects of MLN8237 and MK-5108 were weak for most sites (Figure 6E). We concluded that a decrease in Aurora-A levels or dissociation of N-MYC/Aurora-A complexes promotes stable association of TFIIIC and RAD21 with N-MYC-bound loci in S phase; it is also possible that inhibition of Aurora-A–dependent TFIIIC2 phosphorylation contributes to the increase. Notably, chromatin association of TFIIIC also increased in response to CD532 on sites where no N-MYC peak was detected in ChIP sequencing (Figure S6E). It is possible, therefore, that Aurora-A inhibits TFIIIC binding to chromatin also when complexed by other transcription factors. However, removing the inherent threshold in peak-calling algorithms revealed the presence of N-MYC at the majority (26,165 of 36,736) of all TFIIIC sites detectable after CD532 treatment (Figure S6F). Most likely, therefore, N-MYC is associated with the majority of all TFIIIC sites.

(B) Quantification of the PLA shown in (A). The percentage of cells in S phase is indicated in parallel. Error bars show SD of technical triplicates from one representative experiment (n = 3).

(C) Representative FACs profiles of propidium iodide (PI)-stained cells documenting cell cycle distribution at the indicated times after release from a double thymidine block.

(D) Representative pictures from PLAs documenting complex formation between N-MYC and RAD21 and TOP2A and TFIIIC5 in IMR-5 cells at the indicated times after release from a double thymidine block. Nuclei were stained using Hoechst. Red dots show PLA signals (n = 3).

(E) Quantification of PLAs shown in (A) and (D). Bars show mean + SD of technical triplicates from one representative experiment (n = 3). *p < 0.01, **p < 0.001.

(F) ChIP of IMR-5 cells documenting chromatin association of N-MYC, RAD21, and TFIIIC5 at the indicated gene loci at the indicated times after release from a double thymidine block. Error bars show SD of technical triplicates from one representative experiment (n = 3).

See also Figure S5. n indicates the number of independent biological replicates for each experiment.
Figure 6. Role of Aurora-A in Dynamics of N-MYC Complexes during the Cell Cycle

(A) Immunoblot documenting levels of the indicated proteins and of Aurora-A, which is autophosphorylated at T288 (indicating catalytically active Aurora-A), in IMR-5 MYCN-amplified neuroblastoma cells after 4 hr (left) or 24 hr (right) exposure to 1 μM of the indicated Aurora-A inhibitors (n = 4).

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Cell Cycle-Dependent Regulation of Pause Release by Aurora-A

To understand the role of TFIIIC in N-MYC-dependent pause release of Pol II, we analyzed SH-EP neuroblastoma cells, which express an N-MYCER chimeric protein that can be activated by addition of 4-Hydroxytamoxifen (4-OHT). We engineered these cells to express a doxycycline-inducible shRNA targeting TFIIIC5 (Figure 7A) and performed ChIP sequencing with antibodies directed against total Pol II before and 5 hr after addition of 4-OHT. Inspection of individual genes (ODC1 and NPM1) (Figure S7A) and global analyses (Figures 7B and 7C; Figure S7B) showed that activation of N-MYC led to a strong decrease in Pol II occupancy at the transcription start site and a corresponding decrease in the Pol II “traveling ratio,” which is defined as the ratio of Pol II occupancy at the promoter to occupancy in the gene body (Rahl et al., 2010). Depletion of TFIIIC5 by itself caused a moderate increase in Pol II promoter occupancy and Pol II traveling ratio (Figures 7B and 7C; Figures S7A and S7B). Intriguingly, depletion of TFIIIC5 abrogated the effect of N-MYCER on Pol II (Figures 7B and 7C; Figures S7A and S7B).

To understand which of N-MYC’s effects on Pol II depend on TFIIIC, we performed ChIPs using antibodies that specifically recognize hypo-phosphorylated Pol II (8WG16) (Stock et al., 2007) or Pol II phosphorylated at Ser5, which occurs during escape of Pol II from the promoter, or at Ser2, which is a hallmark of pause release of Pol II, respectively. Intriguingly, ChIP sequencing showed that activation of N-MYC had no significant effect on chromatin association of hypo-phosphorylated Pol II (Figure S7C). To pinpoint the effect of N-MYC on Pol II function, we analyzed the association of Pol II with several MYC target genes for which Pol II does not decrease strongly at the promoter after N-MYC activation. Consistent with the ChIP sequencing data, neither activation of N-MYC nor depletion of TFIIIC5 had a significant effect on the association of hypo-phosphorylated Pol II with the transcription start site of several N-MYC target genes (Figure 7D). In contrast, depletion of TFIIIC5 abrogated both an N-MYC-dependent increase of Pol II that is phosphorylated at Ser5 at the transcription start site and of Pol II phosphorylated at Ser2 at the transcription end site (Figure 7D). Collectively, the data argue that TFIIIC5 is required for N-MYC-dependent escape of Pol II from the promoter and the subsequent pause release of Pol II.

To understand whether N-MYC-dependent pause release is cell cycle-regulated and whether Aurora-A affects this process, we activated N-MYCER in cells synchronized in S phase by a cell cycle-regulated and whether Aurora-A affects this process, with inactive N-MYCER, arguing that the effects on Pol II are mediated via N-MYC (Figure 7E; Figure S7D). CD532 also enhanced association of Pol II with the promoter in a N-MYC-dependent manner and, as consequence, did not alter the overall traveling ratio (Figure S7E). Furthermore, CD532 increased chromatin association of pSer2-Pol II in the gene body and at the transcription end site, and the effect was stronger in the presence of active N-MYC (Figure 7E). We concluded that association with Aurora-A antagonizes N-MYC-dependent promoter binding of Pol II and transcriptional elongation during S phase.

Finally, we explored a possible effect of enhanced transcriptional elongation on DNA replication and monitored phosphorylation of the single-strand DNA binding protein RPA32 at S33, which is phosphorylated by the ATR kinase in response to replication stress (Figure S7F; Liu et al., 2012). Inhibition of Aurora-A during the release of IMR-5 cells from a double thymidine block markedly enhanced phosphorylation of this site. In contrast, we did not observe phosphorylation of RPA32 at S4 and S8, target sites of DNA-dependent protein kinase (DNA-PK), and no activation of CHK1 and stabilization of p53 (Figure S7F). Consistently, inhibition of Aurora-A perturbed S phase progression, but did not prevent S phase entry or induce apoptosis (Figure S7G; Discussion). A model summarizing our findings is shown in Figure 7G.

DISCUSSION

We performed a proteomic analysis of N-MYC complexes in neuroblastoma cells and confirmed previously identified interactions of MYC and N-MYC proteins with MAX, the NuA4 complex, BPTF, p400, and the PAF1 complex. We also validated TFIIIC, RAD21, and TOP2A as interaction partners of N-MYC, arguing that N-MYC uses multiple effector proteins to regulate transcription. Previous proteomic analyses demonstrated the presence of TFIIIC and TOP2A in MYC complexes, suggesting that these interactions are conserved with MYC proteins (Koch et al., 2007).

Our data provide insights into how MYC accelerates several transitions of Pol II from one intermediate promoter state to the
Figure 7. Aurora-A Suppresses N-MYC-Dependent Pause Release of Pol II in S phase

(A) Immunoblot showing levels of TFIIIC5 in SH-EP-N-MYCER cells expressing a Dox-inducible shRNA targeting TFIIIC5 (n = 3). Dox (1 µg ml⁻¹) was added for 30 hr; EtOH was used as a control.

(B) Metagene plot of all expressed genes (n = 14,650) illustrating distribution of Pol II within transcribed regions before and 5 hr after activation of N-MYCER in cells expressing Dox-inducible shTFIIIC5.

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TFIIC is both a general transcription factor of RNA polymerase III and an architectural protein (Van Bortle et al., 2014) and has linked to the function of both cohesin and condensin complexes. In yeast, TFIIC is required for loading condensin complexes onto tRNA genes (D’Ambrosio et al., 2008). In Drosophila, TFIIC co-localizes with RAD21/cohesin at ETC sites (Van Bortle et al., 2014). Our ChIP sequencing data showed that N-MYC co-localizes with TFIIC at multiple promoters and intergenic sites. TFIIC is essential for transcription by RNA polymerase III, but the precise function of TFIIC at ETC sites is unknown. We found that TFIICS promotes association of RAD21 with N-MYC-bound promoters and is required for N-MYC-dependent phosphorylation of Pol II at Ser5 and Ser2 at several target genes, arguing that it promotes both promoter escape and pause release of Pol II. ChiP experiments suggest a hierarchy in which N-MYC promotes binding of TFIIC, and TFIIC, in turn, recruits RAD21. Because RAD21 and cohesin can promote pause release of Pol II (Schaaf et al., 2013), we suggest that N-MYC-dependent recruitment of RAD21 via TFIIC facilitates promoter escape and pause release of Pol II in an enhancer-dependent manner. The model is consistent with the recent observation that depletion of RAD21 represses expression of N-MYC and MYC target genes (Robban et al., 2017). Because the identity of active enhancers is characteristic for each cell, a model in which N-MYC facilitates the function of pre-existing enhancers could explain how N-MYC can “amplify” a pre-existing gene expression program.

Topoisomerase I is activated at core promoters via direct association with Pol II and promotes pause release because it relieves the torsional stress that builds up during early stages of transcription (Baranello et al., 2016). Because TOP2A associates with promoters of highly transcribed genes (Kouzine et al., 2013), it is likely that the association with N-MYC similarly relieves torsional stress during early stages of transcription, suggesting that multiple protein-protein interactions enable MYC proteins to facilitate consecutive steps of transcription (Figure 7G).

Unexpectedly, complexes of N-MYC are highly dynamic during the cell cycle. In vitro, Aurora-A competes with binding of TFIIC and RAD21 to the amino-terminus of N-MYC. A secondary binding site for Aurora-A overlaps with MYCBoxIV, which binds to HCF1, and it is possible, therefore, that Aurora-A also competes with HCF1 for binding to N-MYC (Thomas et al., 2016). In vivo, Aurora-A inhibits chromatin binding of TFIIC and RAD21 and interactions of N-MYC and RAD21, TOP2A, and TFIIC in S phase (Figure 7G). N-MYC-dependent pause release is inhibited in S phase, and inhibition of Aurora-A using CD532 partially restores elongation. Why might this regulation be important? Inhibition of Aurora-A during S phase activates the ATR kinase, which monitors replication stress (Hamperl and Cimprich, 2016). Phosphorylation of RPA32 at S33 with no CHK1 phosphorylation, as observed here, has been linked to recovery of collapsed replication forks rather than CHK1-mediated cell cycle arrest and DNA repair (Shiotani et al., 2013). We hypothesize, therefore, that MYC-dependent transcriptional elongation has an inherent potential to cause conflicts with DNA replication and that complex formation of N-MYC with Aurora-A is one of several co-transcriptional mechanisms that prevent such conflicts (Hamperl and Cimprich, 2016). Notably, TFIIC binding was detected at many sites on chromatin at which we did not detect N-MYC, and TFIIC binding was regulated by Aurora-A also at such sites. It is possible, therefore, that Aurora-A also regulates TFIIC chromatin association independently of N-MYC. However, removing the inherent threshold in peak calling revealed the presence of N-MYC at the majority of all TFIIC sites.

Inhibition of Aurora-A shows therapeutic efficacy in multiple MYC- and N-MYC-driven tumor models (Dardenne et al., 2016; Dauch et al., 2016). Similarly, deregulated expression of MYC and N-MYC activates ATR, and inhibition of ATR or CHK1 are being explored as possible therapeutic strategies for MYC- and N-MYC-driven tumors (Cole et al., 2011; Murga et al., 2011). Our findings suggest that MYC-driven tumors are particularly dependent on Aurora-A to avoid transcription/replication conflicts and open the possibility for rational development of therapies targeting these tumors.

EXPERIMENTAL PROCEDURES

Further details and an outline of the resources used in this work can be found in the Supplemental Experimental Procedures.

Cell Culture and Cell Cycle Synchronization

Neuroblastoma cell lines (IMR-5, IMR-32, and SH-EP) were grown in RPMI-1640 supplemented with 10% fetal calf serum and penicillin/streptomycin. For synchronization in S phase, cells were treated for 16 hr with 2 mM thymidine, released for 8 hr into normal medium, and then blocked again (2 mM, 16 hr). For release, cells were washed with PBS before fresh medium was added.

High-Throughput Sequencing

ChiP and ChiP sequencing were performed as described previously (Walz et al., 2014). RNA sequencing was performed as described previously.

(C) 2D kernel density plot showing the ratio of Pol II occupancy at the promoter to occupancy in the gene body (traveling ratio) in cells treated as above for all expressed genes (n = 14,650) before and after 5 hr of N-MYC-activation.

(D) ChiP of SH-EP N-MYCER cells documenting chromatin association of hypo-phosphorylated Pol II and Pol II phosphorylated at Ser5 (pSer5) or Ser2 (pSer2) at the indicated gene loci before and 5 hr after activation of N-MYCER. Occupancy at the transcription start site (TSS) was analyzed for hypo-phosphorylated Pol II and pSer5PolII. Occupancy at the transcription end site (TES) was analyzed for pSer2PolII. Dox (1 μg ml⁻¹) was added for 30 hr; EtOH was used as a control. Error bars show SD of technical triplicates from one representative experiment (n = 3).

(E) Genome browser tracks illustrating chromatin association of Pol II and Pol II pSer2 at the ODC1 locus. ChiP sequencing was performed on cells synchronized in S phase by double thymidine blockade and treated for 2 hr with CD532 where indicated (1 μM).

(F) 2D kernel density plot showing the ratio of Pol II traveling in response to N-MYCER activation in SH-EP cells that were synchronized in S phase by double thymidine block for all expressed genes (n = 14,650) before and after 4 hr of N-MYCER activation.

(G) Model summarizing our findings. We propose that the multiple protein-protein interactions of N-MYC promote sequential phosphorylation events of Pol II and both promoter escape and pause release. The dashed line indicates that effects on pause release and Ser2 phosphorylation could be secondary to changes in Ser5 phosphorylation. E/C indicates that the complex associates with sites on chromatin that contain either an E-box or a CTCF motif. See also Figure S7. n indicates the number of independent biological replicates for each experiment.
In Situ PLA

The PLA was performed using the Duolink In Situ Kit (Sigma-Aldrich) according to the manufacturer’s protocol. Pictures were taken with a confocal microscope (Nikon Ti-Eclipse) at 60× magnification. For quantification, the dots in not less than 300 cells were counted using ImageJ (Wayne Rasband, NIH). Antibodies are listed in the resource table in the Supplemental Experimental Procedures.

Statistical Methods

Statistical significance between experimental groups was determined by Student’s t test or, when means of three or more groups were compared, by one-way ANOVA. Data analysis was performed with Prism5.0 software (GraphPad). p < 0.05 was considered statistically significant.

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA and ChIP sequencing data reported in this paper is GEO: GSE78967.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.11.090.

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AUTHOR CONTRIBUTIONS

G.B., A.C., K.-Y.M., I.R., E.L., O.S., J.H., S.H., J.K., E.P., C.K., and H.M.M. performed the experiments. A.B. and S.W. analyzed the high-throughput data. G.B., L.C., D.S.R., E.W., R.B., and M.E. devised and supervised the experiments; M.E. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Association with Aurora-A Controls N-MYC-Dependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle

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Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Martin Eilers (martin.eilers@biozentrum.uni-wuerzburg.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

Neuroblastoma cell lines (IMR-5, IMR-32, SH-EP) were verified by STR profiling and grown in RPMI-1640 (Sigma-Aldrich and Thermo Fisher Scientific); HEK293TN and PlatE cells were grown in DMEM (Sigma-Aldrich and Thermo Fisher Scientific). Media were supplemented with 10% fetal calf serum (Biochrom) and penicillin/streptomycin (Sigma-Aldrich). All cells were routinely tested for mycoplasma contamination. For starvation experiments, IMR-5 cells were cultivated in serum-free medium for 48 h and then re-stimulated by change to media containing 10% fetal calf serum.

METHOD DETAILS

Transfection and lentiviral infection

For lentivirus production, HEK293TN cells were transfected using PEI (Polyethyleneimine, Sigma-Aldrich). Transfections of siRNAs were performed using RNAiMAX reagent and OptiMEM (Life Technologies) according to manufacturer’s protocol. siRNAs used are listed in reference table. Cells were harvested 48 h after transfection. For production of stable SH-EP cell lines, PlatE cells were transfected with amino-terminal HA-tagged N-MYC expression plasmids. Retroviral supernatants were used for infection of SH-EP cells stably expressing the murine ecotropic receptor in the presence of 4 µg ml⁻¹ polybrene (Sigma-Aldrich) for 24 h. Infected cells were selected with 3 µg ml⁻¹ blasticidin for 2 weeks, clones were picked and expanded. Lentiviruses expressing a shRNA targeting TFIIC5 (targeting sequence: AAGCGCAGCACCTACAATCTACA), TFIIC2 (targeting sequence:
TCCGTagagatgtcattacctA) or N-MYC (targeting sequence: GAGGAGCATTTTGTATACAA) were produced by transfection of pINDUCER-11 plasmid together with the packaging plasmid psPAX.2, and the envelope plasmid pMD2.G into HEK293TN cells. Virus-containing supernatant was harvested 24 h and 48 h after transfection. IMR-5 cells were infected with lentiviral supernatants in the presence of 4 µg ml⁻¹ polybrene (Sigma-Aldrich) for 24 h. shRNA expression was stimulated by addition of doxycycline (1 µg ml⁻¹) for 12 h and cells were FACS-sorted for RFP-positive cells. Cells were harvested 48 h after induction with doxycycline (1 µg ml⁻¹) or ethanol as control.

For CRISPR constructs guide-RNA (gRNA) were designed based on target sequences against TFIIIC5 reported in Hart et al. (Hart et al., 2015). Oligonucleotides were annealed and cloned into the BsmBI-BsmBI sites downstream from the human U6 promoter in the lentiCRISPR v2 plasmid. Lentivirus production and infection was done as described above.

Cells were treated with 0.05 µg ml⁻¹ of puromycin to select resistant cells.

**Sequences of gRNA.**

| gRNA         | Gene specific part TFIIIC5-exons         |
|--------------|------------------------------------------|
| TFIIIC5-gRNA-1 | CCCTGCCAGACGCACAGGGA                     |
| TFIIIC5-gRNA-2 | GCTCATGCTCCGGCCCCGAGA                    |
| TFIIIC5-gRNA-3 | GAATCCATAGGCTGGCCCGAG                   |
| TFIIIC5-gRNA-5 | CATTTGGGACCAGAGATGGGA                    |
| TFIIIC5-gRNA-6 | GAGAACGAGGCAGGCCAGAAAG                  |
| TFIIIC5-gRNA-7 | ATGGTGTCGCGGAGGTACCC                    |
| TFIIIC5-gRNA-8 | ACCGACCAGAGACCCAGCAC                    |

**Clonogenic assay**

IMR-5 and SH-EP cells expressing a doxycycline-inducible shRNA targeting shTFIIIC5 were seeded at low density and treated with doxycycline or ethanol as control until cells were confluent. Cells were fixed by adding 3.7% formaldehyde to the medium for 15 min. After removing the medium dishes were dried over night. 2 ml of 0.1% crystal violet staining solution was added and incubated for 2 h at room temperature. Dishes were washed in a stream of tap water. After washing dishes were inverted and dried over night.
**Immunoblots and immunoprecipitations**

Whole-cell extracts were prepared using NP-40 buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, and a cocktail of protease inhibitors) with three rounds of freeze/thaw cycles or RIPA buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA; 1% Triton X-100, 0.1% Nadeoxycholate, 0.1% SDS) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Lysates were cleared by centrifugation, separated on SDS or Bis-Tris gels and transferred to a PVDF membrane (Millipore). Antibodies are listed in the resource table.

For immunoprecipitation, cells were re-suspended in lysis buffer containing 20 mM HEPES-KOH (pH 7.8), 140 mM KCl, 0.2 mM EDTA, 0.1% NP-40 supplemented with a cocktail of protease and phosphatase inhibitors. After brief sonication, samples were incubated on ice for 30 min and cleared by centrifugation. Co-immunoprecipitation was carried out in lysis buffer using 2 µg of antibodies and 1–2 mg lysate.

**Flow cytometry analysis (FACS)**

BrdU-PI-FACS was performed as described previously (Schulein-Volk et al., 2014). Subconfluent cells were labelled with 20 µM 5-Bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) for 1 h. Cells were harvested together with the supernatant, washed with ice-cold PBS and fixed in 80% ethanol overnight at -20 °C. Cells were washed with cold PBS and incubated in 2 M HCl/0.5% Triton X-100 for 30 minutes at room temperature. Cell pellets were neutralized by incubating with Na₂B₄O₇. The pellet was incubated with Anti-BrdU-FITC antibody diluted in 100 µl 1% BSA, 0.5% Tween-20 in PBS for 30 minutes at room temperature in the dark. After washing with PBS, the cells were re-suspended in PBS with RNAse A (24 µg ml⁻¹) and propidium iodide (PI, 54 µM) and incubated for 30 min at 37 °C.

For PI-FACS cells were harvested by trypsinization, washed with cold PBS and fixed in 80% ethanol overnight at -20 °C. After washing with PBS, the cells were re-suspended in PBS with RNAse A (24 µg ml⁻¹) and PI (54 µM) and incubated for 30 min at 37 °C. Subsequent analysis was performed on a BD FACSCanto II flow cytometer using BD FACSDIVA™ Software.
**Mass spectrometric analysis**

Proteins from immunoprecipitations were dissolved in NuPAGE LDS sample buffer (Life Technologies), reduced with 50 mM DTT at 70 °C for 10 min, alkylated with 120 mM iodoacetamide at room temperature for 20 min and separated on NuPAGE Novex 4-12% Bis-Tris gels (Life Technologies) with MOPS buffer according to manufacturer’s instructions. Gels were stained for 45 min with Simply Blue™ Safe Stain (Life Technologies). Each gel lane was cut into 15 bands, gel bands were chopped and destained with 70% acetonitrile in 100 mM NH₄HCO₃ (pH 8), shrunk with 100% acetonitrile and dried in a vacuum concentrator (Concentrator 5301, Eppendorf). Dried gel pieces were suspended in 100 mM NH₄HCO₃ (pH 8) containing 0.1 μg trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) and proteins were digested overnight at 37 °C.

NanoLC-MS/MS analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Scientific) equipped with an EASY-Spray Ion Source and coupled to an EASY-nLC 1000 (Thermo Scientific). Peptides were loaded on a trapping column (2 cm x 75 μm ID, PepMap C18 3 μm particles, 100 Å pore size) and separated on an EASY-Spray column (25 cm x 75 μm ID, PepMap C18 2 μm particles, 100 Å pore size) with a 30-minute linear gradient from 3% to 30% acetonitrile and 0.1% formic acid. MS scans were acquired in the Orbitrap analyser with a resolution of 30,000 at m/z 400, MS/MS scans were acquired in the Orbitrap analyser with a resolution of 7,500 at m/z 400 using HCD fragmentation with 30% normalized collision energy. A TOP5 data-dependent MS/MS method was used. Lock mass option was applied for internal calibration in all runs using background ions from protonated decamethylcyclopentasiloxane (m/z 371.10124).

Mascot Distiller 2.4 (Matrix Science) was used for raw data processing and for generating peak lists with standard settings for the Orbitrap Velos. Mascot Server 2.4 was used for database searching with the following parameters: peptide mass tolerance: 8 ppm, MS/MS mass tolerance: 0.02 Da, enzyme: “trypsin” with three missed cleavage sites allowed for trypsin, fixed modification: carbamidomethyl (C), variable modifications: Gln->pyroGlu (N-term. Q) and oxidation (M). Database searching was performed against UniProt human
database. Spotfire was used to visualize data. Protein scores were calculated using MaxQuant (Cox and Mann, 2008).

In vitro binding assays

Constructs encoding FLAG-tagged fragments of the N-MYC transactivation domain were cloned into pETM6T1 and expressed as His-NusA fusions. These were purified on 5 ml chelating Sepharose columns (GE Healthcare) charged with nickel; the His-NusA tags were cleaved away using TEV Nla protease and removed by nickel affinity chromatography and the FLAG-N-MYC fragments were further purified by gel filtration using a Superdex 75 column (GE Healthcare) into N-MYC fragment buffer (20 mM HEPES pH 7.5, 100 mM NaCl). For in vitro pull-down assays from HeLa cell lysate, recombinant N-MYC fragments were immobilised on 30 μl Anti-FLAG M2 affinity gel (Sigma-Aldrich) and incubated with 1.2 mg of HeLa cell lysate in 150 mM sodium chloride, 50 mM Tris pH 8.0, 1.0% NP-40 and 1x Roche complete protease inhibitors. The final volume of the mix was made up to 495 μl with N-MYC fragment buffer. The concentration of FLAG-N-MYC fragments was determined by absorbance at 280 nm, however adjustments were made to account for differences observed in apparent concentration by immunoblots. The final protein concentrations were 9.3 μM for all fragments apart from N-MYC 1-137 (6.2 μM) and 46-89 (14.3 μM). The mixture was incubated by slow rotation at 4 °C for two hours prior to washing the gel three times with 500 μl N-MYC fragment buffer. FLAG-tagged fragments were eluted by addition of 85 μl of N-MYC fragment buffer with 0.25 mg ml⁻¹ 3x FLAG-peptide (Sigma-Aldrich). This mix was allowed to rotate at 4 °C for 30 minutes prior to collection of eluates. Mixes and eluate fractions were subjected to SDS-PAGE and subsequently analyzed by immunoblotting. The competition assays were performed as described above with the exception that mixtures were spiked with either recombinant Aurora-A kinase or an equivalent amount of Aurora-kinase buffer. The recombinant Aurora-A protein used comprised residues 122-403 and C290A:C393A (Burgess and Bayliss, 2015). Expression and purification was performed as previously described (Bayliss et al., 2003). The protein was finally buffer exchanged by
repeated dilution and concentration into 150 mM NaCl, 20 mM HEPES pH 7.5, 5 mM MgCl₂, and 10% glycerol.

High-throughput sequencing

ChIP and ChIP-sequencing was performed as described previously (Walz et al., 2014). Cells were treated with 1% formaldehyde for 10 min at room temperature following 5 min of incubation with glycine. After cell lysis (5 mM PIPES pH 8.8, 5 mM KCl, 0.5% NP40), nuclei were re-suspended in RIPA buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1% Triton-X-100, 0.1% deoxycholic acid (DOC), 0.1% SDS, 1 mM EDTA containing protease inhibitor cocktail) and DNA was fragmented to a size <500 bp using a Branson sonifier. Antibodies were bound to Protein A/G-dynabeads (Invitrogen) and immunoprecipitated. Chromatin was eluted with 1% SDS and crosslinking was reverted overnight. Chloroform/phenol extraction was used for purification. ChIP-sequencing was performed as described before (Chen et al., 2008). Purified DNA was end-repaired, A-tailed, ligated to Illumina adaptors, size-selected (200 bp) and purified with Qiagen gel extraction kit. DNA fragments were amplified by 15-18 cycles of PCR and library size was tested with the Biorad Experion system. The amount of library DNA was quantified using a picogreen assay and subjected to Illumina GAIIx or Illumina NextSeq 500 sequencing according to the manufacturer’s instructions. After base calling with the Genome Analyzer Data Collection Software, high quality PF-clusters (according to the CASAVA filter) were selected for further analyses. Antibodies are listed in the resource table. All ChIP-sequencing experiments were performed 1-3 times and results validated with independent ChIPs on individual genes.

Sequences of oligonucleotides for qPCR.

|          | forward                        | reverse                        |
|----------|--------------------------------|--------------------------------|
| BIRC5 TSS| CTTTGAAAGCAGTCGAGGGG          | TGTGCCCCGGGAGTTGTAGTC          |
| BIRC5 TES| GGTCTGTGTGGAGGGTGA            | GAAGTCAAGGCCAGTTTG             |
| CDK14    | CCCTCTTCTTCAATCCATCA          | CGGAGCAGCAGATCTGTAGT           |
| CLINT1   | GGCACTCTCAACGGTTTCTT          | AAATTATTGGGGGAGGGGCG           |
| EIF2B5   | TTTTCGTCGCCGACCCTAAC          | CTGAGAGCTGTTTGCCACGTG          |
| EIF3A    | GAGAGGAGAGAGAGGGGAAC          | GCTCCTTCTTCTCGTCCTCT           |
| EIF4H    | CAGCTCTCAGGGTCACCTC           | CTACGGCCGCCATTAGGTG            |
| ERCC     | CCTCACTATCATCCATCCGCT         | AGGTTCCCAGGCCTACTC             |
| Gene          | Region          | Sequence 1          | Sequence 2          |
|--------------|-----------------|---------------------|---------------------|
| GALNT14      | (N-MYC binding) | AATGTGCTCGTCCCTACCACA | AGTAGCCAGGCAAGTGAACC |
| GALNT14      |                 | CTAGACCCAGGATCCGGTTG | CAGGCTCGCTTCTCTTCGA |
| GLN3 TSS     |                 | GTGACGGCTGTCAGTGG   | CATATTGGCTGAGAAGAGAC |
| GLN3 TES     |                 | GTTATGTCATGCACTGTGG | CTACTCCACTCAATAGGATG |
| Intergenic   | region (chr3)   | TATGTTGCTGTCACCACCACAT | TATCTGTGTAGGCAGGCTG |
| Intergenic   | region (chr5)   | GAGGCCAGTGGAAAGAGACA | TCTTTAACCCACTGCCACCT |
| Intergenic   | region (chr6)   | GGGCTTGGATATGCCGGTGTA | CCTCTTTCCTGTATATGCTCC |
| Intergenic   | region (chr14)  | CTTTCTCTCTCTTCAGCTCC | CTCTCTGCGCTTCTTCCTCA |
| Intergenic   | region (chr16)  | GGAAGACACCTGGTGGCAAG | TCACAGGCAGATGTTAGGC |
| Intergenic   | region (chr21)  | CTTTCCCCAGGCCGCCATC  | GGCATCCCGAGTCAAGAC |
| Intergenic region (chr3) |             | GTGATTTTCAAGCCCTCCGCGT | GTGATTTTCAAGCCCTCCG |
| LDHA TSS     |                 | GAGGAGGCAGCCAGCTACTTA | GTGGAACAGTCAGTGGCTCC |
| LDHA TES     |                 | GTTGGACACAGGCAGATCTTCT | GTTGGACACAGGCAGATCTTCT |
| LHFPL        |                 | GCCATGCCCTCAGTACTCTCT | GAACCTCAGTCAGTGCCAC |
| MEI4         |                 | GCCATGCCCTCAGTACTCTCT | TAAGACEACACACGCAGATAG |
| METAP1       |                 | AAGGAGGCAGATGTTGAACT | TCTTACTGACGAGACACCA |
| Negative     | region (chr1)   | GCAGTTCAACACTCAAGCCAATAGAC | CACAAATTACGGCACTTGGCAG |
| Negative     | region (chr11)  | TTTTCTCACATTGCCCTGT  | TCAATGCTGTACCAGGCAAA |
| NCL TSS      |                 | CTACACCACCTCCTGAAATCC | TTGTCTCGCTGGGAAAGG |
| NCL TES      |                 | AGCCCTTCATCCAGGTTGAGAA | GGCCACAGGCCATATAGAC |
| NME1 TSS     |                 | GGGTGGAGAGGAAAGAAAAC | TGGGTAGGCACTGACTCT |
| NME1 TES     |                 | GATGGCTAGGGTGTCCGGAG | AGCACTACTGAGGCTGAGT |
| NPM1 TSS     |                 | TCCAGCCGGAGGCGATGG   | CACGCGAGTAACTCAGG |
| NPM1 TES     |                 | TAGGCGCTGGGGTCTTCTTTTCTT | AACTGAGGCTCTACAGCC |
| PLK1         |                 | GTTTGAATTCCGGGGAGGAC | CAGTCACTGCAGCACTCATG |
| PPRC1        | (N-MYC binding) | GAAGGCTGAGACCTCCATG | GTTCTCCGGGAAATGTGCT |
| PPRC1        |                 | GTGAGATACGCGTCTGAGAG | TGCTGACGCTTTCTTCA |
| RCC1 TSS     | (N-MYC binding) | AGTTGGTCTGCTTCTCTTCTT | GCATTAGACCCCAACTCCG |
| RCC1 TES     |                 | GTAGCTGGAGACTGGGAGTG | TTAGGCGCATGGTGGTAG |
| tRNA119Ala   |                 | ACTGTGCGCCAGGAGATGAG | AATCTACGTGACTCGCTT |
| tRNA7Leu     |                 | ATGTAGCATAAGCGGCCTAG | ACTGTCAGGAGTGCTT |

RNA-sequencing was performed as described previously (Jaenicke et al., 2016) using an Illumina NextSeq 500. RNA was extracted using RNeasy mini columns (Qiagen) including on-column DNase I digestion. mRNA was isolated using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB) library preparation was performed with the NEBNext®
Ultra™ RNA Library Prep Kit for Illumina following the instruction manual. Libraries were size-selected using Agencourt AMPure XP Beads (Beckman Coulter) followed by amplification with 12 PCR cycles. Library quantification and size determination was performed with the Experion Automated Electrophoresis System (Bio-Rad).

**Peptide microarrays**

For evaluation in microarray format Myc peptides were synthesized in parallel using a ResPep SL synthesis robot (Intavis AG) equipped with a Celluspot synthesis module and printed using a slide spotting robot (Intavis AG). Total synthesis time was 256 h. Coupling reagents were freshly prepared every 48 h. Synthesis was based on Standard Fluorenlymethoxycarbonyl (Fmoc) peptide synthesis using reagents from Sigma-Aldrich and Iris and performed on acid-soluble Fmoc-β-Alanine etherified cellulose disks (area 0.12 cm², loading 1.0 μmol cm⁻²). N-terminal Fmoc protection was removed by adding 2 μl and 4 μl 20% Piperidine in N-Methyl Pyrrolidone (NMP) for 5 and 10 min. Four couplings (10, 20, 30 and 40 min) using Oxyma/N,N′-Diisopropylcarbodiimide/Amino Acid in the relation (1.1/1.5/1.0) in at least 5-fold excess followed by two washing steps (100 μl and 300 μl NMP) and 4 μl capping solution (5% Acetic Anhydride in NMP) achieved peptide elongation by one amino acid. The subsequent peptide work-up was performed manually on all peptides in parallel after transfer of the cellulose disks into 96 deep-well blocks. Peptide side-chain deprotection was achieved with 150 μl deprotection solution (trifluoroacetic acid/triisopropylsilane/water/DCM: 80%, 3%, 5%, 12%) for 2 h. Disks were then solubilized overnight in 250 μl of cellulose solvation solution (trifluoroacetic acid/trifluoromethansulfonic acid/triisopropylsilane/water: 88.5%, 4%, 2.5%, 5%) under strong agitation. 750 μl Diethylether (-20° C) was added to the dissolved cellulose−peptide conjugates. The mixture was briefly agitated and kept at -20 °C for 1 h. Precipitated conjugates were pelleted by centrifugation at 2,000 rcf for 30 min at 4 °C. After removal of the supernatant the pellet was additionally washed twice with 750 μl fresh Diethylether (-20° C). After the final washing step, residual ether was evaporated and 250 μl of dimethyl sulfoxide (DMSO) was added to re-
solvate the cellulose-peptide conjugates. The cellulose-peptide conjugate stock solutions were stored at -20 °C. Prior printing 80 µl of the stocks were transferred to a 384-well plate, and mixed with 20 µl SSC buffer (150 NaCl; 15 µM Na₃C₆H₅O₇; pH 7.0). 50 nl of each peptide was contact printed on coated glass slides with a slide spotting robot (Intavis AG).

After drying overnight, peptide microarrays were washed, equilibrated and blocked with 2 x 2 ml array buffer (0.01 M phosphate buffered saline (NaCl 138 mM; KCl 2.7 mM); pH 7.4, 0.05% bovine serum albumin). Arrays were incubated with 2 ml Aurora-A kinase at 4 °C for 1 h. After washing with 4 x 2 ml array buffer the arrays were incubated with 2 ml array buffer and horse radish peroxidase (HRP) coupled anti-His antibody (Thermo Fisher Scientific, MA1-21315-HRP, dilution 1:20,000). Aurora-A kinase binding was visualized after washing with 4 x 2 ml array buffer with 300 µl ECL Western Blotting Substrate (Thermo Fisher Scientific). Prepared microarrays were imaged using the GeneSys Pxi system (Syngene). The resulting images were analyzed using the Active Motif software. For all peptide array data sets >95% of the peptide SPOTs were within an <5% error margin when comparing intensities between peptide duplicates.
QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatic analyses and statistics

Base calling was performed with Illumina’s CASAVA software or FASTQ Generation software v1.0.0 and overall sequencing quality was tested using the FastQC script. For ChIP-sequencing, fastq files were mapped to the human genome (hg19) using Bowtie v1.1.1. (Langmead and Salzberg, 2012) with default parameters and normalized to the sample with the smallest number of mapped reads. Peaks were called using MACS v1.4.2 (Zhang et al., 2008) with a p-value cut-off of $1.0 \times 10^{-6}$ (N-MYC, TFIIIC5), $1.0 \times 10^{-11}$ (RAD21) or $1.0 \times 10^{-12}$ (CTCF) and the input sample as control. Wiggle files were generated using MACS, bedGraph files were generated using the genomecov function from BEDTools and the Integrated Genome Browser (Nicol et al., 2009) was used to visualize density files. Heat maps illustrating DNA binding were calculated using DeepTools (Ramirez et al., 2016) with a resolution of 50 bp. Overlapping N-MYC/TFIIIC binding sites were determined using the intersectBed function from BEDTools (Quinlan, 2014) with a minimum overlap of 1 bp and corresponding p-values were calculated using a permutation test with $1.0 \times 10^{6}$ iterations.

Genes were assigned to be bound by N-MYC or TFIIIC, if a peak was called within a region of +/-1 kb around a transcriptional start site.

N-MYC binding sites were generated by intersecting N-MYC peaks in DMSO and CD532 using bedtools with a minimum overlap of 1 bp leading to 10,157 peaks. TFIIIC5 reads in +CD532 sample were counted in a region of +/-100 bp around the N-MYC peak summit and binding sites with less then 8 reads were defined as N-MYC-only sites. Only binding sites that were within +/-1 kb around a transcriptional start site of an RNAPII transcribed gene were considered. To determine the occupancy of N-MYC, TFIIIC5, CTCF and RAD21 the number of reads was counted in a region of +/-100 bp around the N-MYC peak summit.

Changes in N-MYC and TFIIIC occupancy upon CD532 treatment at N-MYC/TFIIIC peaks was calculated by counting tags in a region of 50 bp around the N-MYC peak, p-values were calculated using a two-tailed one-sample Wilcoxon signed-rank test. Occupancy at promoters of selected gene sets was measured by counting tags in a region of +/-0.5 kb
around the TSS and corresponding p-values were calculated by a two-tailed, paired Wilcoxon signed-rank test.

For de novo motif analyses of N-MYC and TFIIIC peaks the MEME and DREME algorithms implemented in the MEME Suite (Bailey et al., 2009) were used with an input region of +/- 50 bp around the peak summit. To compute occurrences of pre-defined motifs (E-box: CACGTG, CTCF: MA0139.1, AP2a: MA0003.2) the CENTRIMO tool was used: the frequency of a motif at a certain position was normalized to the number of input sequences and a rolling mean of 20 bp was applied for smoothing the curves.

RNAPII occupancy was calculated by counting reads in promoters (-30 bp to +300 bp relative to TSS), gene bodies (+300 bp to TES) and TES (TES to +1 kb), addition of one pseudocount/kb and normalization to region length. The traveling ratio is defined as occupancy in promoter divided by occupancy in gene body (Rahl et al., 2010). For the analyses only expressed/RNAPII-bound genes were used (2D Kernel density plots: >20 RNAPII counts/kb in promoters; bin plots: log$_2$CPM >1.28). 2D Kernel density plots were generated with the smoothScatter function in R and default settings, bin plots were generated by calculating the mean of equal-sized bins. Metagene window plots were produced using ngs.plot (Shen et al., 2014) and all annotated genes from the UCSC RefSeq list.

For RNA-sequencing, reads were mapped to hg19 with TopHat2 (Kim et al., 2013) and Bowtie1 with default settings. Reads per gene were counted using the countOverlaps function from the R package GenomicRanges. Weakly expressed genes (mean count over all samples <1.5) were removed and differentially expressed genes were called using EdgeR. For Venn diagrams, genes were filtered based on log$_2$FC threshold of |0.5| (siTFIIIC5 vs siCtr) or |1.0| (siRAD21 vs siCtr). Statistical significant overlap of regulated genes presented in Venn diagrams were calculated using a Monte-Carlo simulation with 100,000 iterations and 17,450 genes as population size. The p-values were calculated as $(r+1)/(n+1)$ with $r$ as the number of iterations producing a greater or equal overlap than the actual one and $n$ as the total number of iterations (North et al., 2002). GSE analyses (Subramanian et
were performed with signal2noise metric, 1,000 permutations and the C2 gene set collection of MSigDB. To measure gene expression changes in N-MYC depleted neuroblastoma cells (GSE39218, samples with double infection) raw data were downloaded, RMA-normalized, probes to genes collapsed by mean and log₂ fold changes were calculated. Association of gene expression of specific gene sets in human neuroblastoma was determined as described previously (von Eyss et al., 2015). Expression data at different stages were downloaded from GEO (GSE16476), RMA-normalized and the association score was calculated using a custom R script using the following formula: -log₁₀ p-value x direction. Here, -1 means a negative and +1 a positive association. Box and whisker plots are characterized by a horizontal line reflecting the median, boxes spanning the first and third quartile and whiskers expanding to 1.5x interquartile range of the first and third quartile, respectively. Outliers are shown as individual dots, p-values comparing medians in box plots are calculated with one- or two-sample two-tailed Wilcoxon Signed-rank tests. For binned data the mean of each bin is plotted.

Statistical significance between experimental groups were determined by Student’s t test or, when means of three or more groups were compared, by one-way ANOVA. Data analysis was performed with Prism5.0 Software (GraphPad). P values < 0.05 were considered statistically significant.

DATA AND SOFTWARE AVAILABILITY

ChIP- and RNA-sequencing datasets are available at the Gene Expression Omnibus under the accession number GEO: GSE78957. The authors do not declare a conflict of interest.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Actin beta (mouse)  | Sigma-Aldrich | Clone: AC15 Cat# A5441 |
| Aurora-A/ALK antibody (rabbit) | Cell Signaling | Cat# 3092 |
| Aurora-A (rabbit)   | Genetex | Clone: C3 Cat# GTX104620 |
| Aurora-A (mouse)    | Sigma-Aldrich | Clone: 35C1 Cat#A1231 |
| Aurora-A (goat)     | Santa Cruz | Clone: N-20 Cat# sc-14318 |
| pT288 Aurora-A (rabbit) | Cell Signaling  | Cat# 2914 |
| ATM pS1981 (mouse)  | Millipore | Clone: 10H11.E12 Cat# MAB3806 |
| CDK2 (rabbit)       | Santa Cruz | Clone: M-2 Cat# sc-163 |
| CTCF (rabbit)       | Abcam | Cat# ab70303 |
| FLAG-tag (mouse)    | Sigma-Aldrich | Clone: M2 Cat# F1804 |
| HA-tag (rabbit)     | Abcam | Cat# ab9110 |
| N-MYC (mouse)       | Santa-Cruz | Clone: B8.4.B Cat# sc-53993 |
| N-MYC (mouse)       | Calbiochem | Clone: NCM II 100 Cat# OP13 |
| P400 (rabbit)       | Abcam | Cat# ab5201 |
| RAD21 (rabbit)      | Bethyl | Cat# A300-080A |
| TFIIIC1 (mouse)     | Santa Cruz | Clone: F-12 Cat# sc-398780 |
| TFIIIC2 (mouse)     | Abcam | Cat# ab89113 |
| TFIIIC5 (rabbit)    | Bethyl | Cat# A301-242A |
| TRRAP (rabbit)      | Abcam | Cat# ab73546 |
| TOP1 (rabbit)       | Bethyl | Cat# A302-589A |
| TOP2A (rabbit)      | Bethyl | Cat# A300-054A |
| TOP2B (rabbit)      | Bethyl | Cat# A300-949A |
| Tubulin beta (mouse)| Millipore | Clone: TU-20 Cat# MAB1637 |
| Vinculin (mouse)    | Sigma-Aldrich | Clone: vVin-1 Cat# V9131 |
| RPA32 (mouse)       | Santa Cruz | Clone: MA34 Cat# sc-53496 |
| Antibody Description                          | Supplier          | Catalog Number | Clone/Tag Information                          |
|----------------------------------------------|-------------------|----------------|-----------------------------------------------|
| RPA32 pS33 (rabbit)                          | Bethyl            | Cat# A300-246A |                                               |
| RPA32 pS4/8 (rabbit)                         | Bethyl            | Cat# A300-245A |                                               |
| CHK1 (rabbit)                                | Santa Cruz        |                | Clone: FL-476 Cat# sc-7898                     |
| pS345 CHK1 (rabbit)                          | Cell Signaling    |                | Clone: 133D3 Cat#2348                        |
| P53 (mouse)                                  | Millipore         |                | Clone: pAb1801 #Cat OP09                       |
| BRCA1 (rabbit)                               | Bethyl            | Cat# A300-000A |                                               |
| RPA70 (mouse)                                | Millipore / Calbiochem |        | Clone: RPA34-20 Cat NA19L                     |
| CDC73 (rabbit)                               | Bethyl            | Cat# A300-170A |                                               |
| CTR9 (rabbit)                                | Bethyl            | Cat# A301-395A |                                               |
| TRRAP (rabbit)                               | Bethyl            | Cat# A310-373A |                                               |
| FITC anti-BrdU, Mouse IgG1, kappa            | Biozol / BioLegend|                | Clone: 3D4 Cat BLD-364104                     |
| RNA polymerase II (rabbit)                   | Santa Cruz        |                | Clone: N-20 Cat sc-899                        |
| RNA polymerase II CTD repeat YSPTSPS (phospho Ser2) (rabbit) | Abcam         | Cat# ab5095                             |                                               |
| RNA polymerase II (hypo-phosphorylated) (mouse) | Santa Cruz        |                | Clone: 8WG16 Cat sc-56767                     |
| RNA polymerase II (phospho Ser5) (mouse)     | BioLegend / previous Covance |          | Clone: CTD4H8 Cat MMS-128P                     |
| Anti-rabbit HRP                              | Amersham          | Cat# NA934     |                                               |
| Anti-mouse HRP                               | Amersham          | Cat# NA931     |                                               |
| Rabbit TrueBlot                              | Rockland          | Clone: eB182 Cat 18-8816-33               |
| Mouse TrueBlot                               | Rockland          | Clone: eB182 Cat 18-8816-33               |
| IRDye® 800CW Donkey anti-Mouse IgG (H + L)    | LI-COR Biosciences GmbH | Cat# 926-32212 |                                               |
| IRDye® 800CW Donkey anti-Rabbit IgG (H + L)   | LI-COR Biosciences GmbH | Cat# 926-32213 |                                               |
| 6x-His Tag Monoclonal Antibody (mouse)       | Thermo Fisher Scientific |    | Clone: HIS.H8 Cat MA1-21315                   |

**Chemicals, Peptides, and Recombinant Proteins**

| Chemical Name               | Supplier          | Catalog Number |
|-----------------------------|-------------------|----------------|
| MK-5108                     | Selleckchem       | Cat# S2770     |
| MLN8237                     | Selleckchem       | Cat# S1133     |
| CD532                       | R. Bayliss        | N/A            |
| Item                                                      | Supplier          | Catalog #       |
|-----------------------------------------------------------|-------------------|-----------------|
| CD532                                                     | Calbiochem / Merck| Cat# 532605     |
| Thymidine                                                 | Sigma-Aldrich     | Cat# T9250      |
| 3x FLAG-peptide                                          | Sigma-Aldrich     | Cat# F4799      |
| Aurora-A protein                                         | Burgess and Bayliss, 2015 | N/A          |
| Hoechst 33342                                            | Sigma-Aldrich     | Cat# B2261      |
| 4-Hydroxytamoxifen                                       | Sigma-Aldrich     | Cat# H7904-5MG  |
| Puromycin                                                 | InvivoGen         | Cat# ant-pr-1   |
| MG-132                                                   | Calbiochem        | Cat# 474790-20MG|
| Polybrene                                                | Sigma-Aldrich     | Cat# 107689-100G|
| Dynabeads® Protein A                                     | Life Technologies GmbH | Cat# 10002D   |
| Dynabeads® Protein G                                     | Life Technologies GmbH | Cat# 10004D   |
| Pierce® Anti-HA Agarose                                  | Pierce            | Cat# 26181      |
| Pierce® HA Peptide                                       | Pierce            | Cat# 26184      |
| Formaldehyde (37%)                                       | Roth              | Cat# 4979.1     |
| Crystal Violet                                           | Sigma-Aldrich     | Cat# C0775-25G  |
| Critical Commercial Assays                               |                   |                 |
| Duolink® In Situ PLA® Probe Anti-Rabbit PLUS, Affinity purified Donkey anti-Rabbit IgG (H+L) | Sigma-Aldrich     | DUO92002        |
| Duolink® In Situ PLA® Probe Anti-Mouse MINUS, Affinity purified Donkey anti-Mouse IgG (H+L) | Sigma-Aldrich     | DUO92004        |
| Duolink® In Situ Detection Reagents Red                  | Sigma-Aldrich     | DUO92008        |
| Duolink® In Situ Wash Buffers, Fluorescence              | Sigma-Aldrich     | DUO82049        |
| Rnase Mini Kit                                           | Qiagen            | 74106           |
| Rnase-free Dnase kit                                     | Qiagen            | 79254           |
| MiniElute PCR Purification Kit                           | Qiagen            | 28006           |
| QIAquick PCR Purification Kit                            | Qiagen            | 28106           |
| QIAquick Gel Extraction Kit                              | Qiagen            | 28704           |
| Experion RNA StdSense kit                                | Bio-Rad           | 700-7103        |
| Experion DNA 1K Kit                                      | Bio-Rad           | 700-7307        |
| NEBNext® Ultra™ RNA Library Prep Kit for Illumina         | NEB               | E7530 L         |
| NEBNext Poly(A) mRNA Magnetic Isolation Module           | NEB               | E7490 L         |
| NEBNext® ChIP-Seq Library Prep Master Mix Set for Illumina® | NEB               | E6240 L         |
| NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1) | NEB               | E7600 S         |
| NextSeq® 500/550 High Output Kit v2 (75 cycles)           | Illumina          | FC-404-2005     |
| Reagent Name                                                      | Manufacturer          | Catalog Number |
|-----------------------------------------------------------------|-----------------------|----------------|
| Quant-iT™ Pico Green®                                            | Thermo Fisher Scientific Inc. | Cat# P7589     |
| ABsolute QPCR SYBR Green Mix (no ROX)                           | Thermo Fisher Scientific | Cat# AB-1158/B  |
| PowerUp™ SYBR® Green Master Mix                                 | Thermo Fisher Scientific | Cat# A25778    |

**Deposited Data**

| Raw and analyzed data                                           | This paper            | GEO: GSE78957 |
|-----------------------------------------------------------------|-----------------------|----------------|
| Raw data N-MYC depleted neuroblastoma cells                     | Valentijn LJ et al., 2012 | GEO: GSE39218 |
| Expression data at different stages of neuroblastoma            | R. Versteeg           | GEO: GSE16476 |

**Experimental Models: Cell Lines**

| Human neuroblastoma: IMR-5                                      | A. Eggert              | N/A            |
|-----------------------------------------------------------------|-----------------------|----------------|
| Human neuroblastoma: IMR-32                                     | M. Schwab              | N/A            |
| Human neuroblastoma: SH-EP                                      | M. Schwab              | N/A            |
| Human neuroblastoma: SH-EP NMYCER                                | M. Eilers              | N/A            |
| Pseudoviral Particle Producer cell line: HEK293TN                | ATCC                   | Cat# CRL-11268 |
| Retroviral Packaging cell line: PlatE                            | B. v. Eyss             | N/A            |

**Recombinant DNA**

| plInducer-11                                                     | Addgene                | Cat# 44363 Meerbrey et al., 2011 |
|-----------------------------------------------------------------|-----------------------|---------------------------------|
| psPAX.2                                                         | Addgene                | Cat# 12260 D. Trono              |
| pMD2.G                                                          | Addgene                | Cat# 12259 D. Trono              |
| pETM6T1                                                         | R. Bayliss             | N/A                             |
| lentiCRISPR v2                                                   | Addgene                | Cat# 52961 Sanjana et al., 2014 |

**Sequence-Based Reagents**

| ON-TARGETplus SMARTpools GTF3C5                                 | GE Healthcare          | L-020031-00-0005 |
|-----------------------------------------------------------------|------------------------|-----------------|
| ON-TARGETplus SMARTpools RAD21                                  | GE Healthcare          | L-006832-00-0005 |
| ON-TARGETplus non-targeting control pool                        | GE Healthcare          | D-001810-10-20  |
| shRNA targeting TFIIIC5: AAGCGGACGCACCTACA                         | (Fellmann et al., 2013) | shRNA ID: GTF3C5.1361 |
| shRNA targeting TFIIIC2: TCCGATGACGATGTACCTA                      | Fellmann et al., 2013  | shRNA ID: GTF3C2.435 |
| shRNA targeting N-MYC: GAGGAGCATGTTTTGTATACAA | Fellmann et al., 2013 | shRNA ID: MYCN.2214 |
|-----------------------------------------------|----------------------|---------------------|
| Primers for ChIP, see Supplemental Experimental Procedures | This paper | N/A |
| gRNA for CRISPR, see Supplemental Experimental Procedures | Hart et al., 2015 | N/A |

**Software and Algorithms**

| Tool                  | Reference                  |
|-----------------------|----------------------------|
| Bowtie v1.1.1         | Langmead and Salzberg, 2012|
| MACS v1.4.2           | Zhang et al., 2008         |
| Integrated Genome Browser | Nicol et al., 2009      |
| SeqMINER              | Ye et al., 2014            |
| BEDTools              | Quinlan, 2014              |
| MEME Suite            | Bailey et al., 2009        |
| TopHat2               | Kim et al., 2003           |
| GSEA                  | Subramanian et al., 2005   |
| DeepTools             | Ramirez et al., 2016       |
| Ngs.plot              | Shen et al., 2014          |
| Prism5.0 Software     | GraphPad                   |
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| Gene name | log₂ Ratio wt vs. Ctr | log₂ Ratio mut vs. Ctr | Reference |
|-----------|-----------------------|------------------------|-----------|
| MYCN      | 10.88                 | 10.31                  | NA        |
| EP400     | 8.99                  | 8.47                   | Fuchs, M. et al., Cell (2001). |
| TRRAP     | 7.75                  | 8.43                   | McMahon, S.B. et al., Cell (1998). |
| MAX       | 7.61                  | 5.65                   | Blackwood, E.M. & Eisenman, R.N., Science (1991). |
| DMAP1     | 5.08                  | 5.68                   | Cai, Y. et al., J Biol Chem (2005). |
| BPTF      | 4.90                  | 3.91                   | Richart, L. et al., Nat Commun (2016). |
| CDC73     | 4.14                  | 4.29                   | Jaenicke, L.A. et al., Mol Cell (2016). |
| VPS72     | 3.70                  | 3.81                   | Cai, Y. et al., J Biol Chem (2005). |
| MYC BP2   | 3.33                  |                        | Guo, Y. et al., PNAS (1998). |
| HCFC1     | 3.56                  | 3.03                   | Thomas, L.R. et al., Oncogene (2016). |
| YEATS4    | 3.33                  | 2.84                   | Piccinni, E. et al., Acta Biochim Pol (2011). |
| EPC1      | 3.08                  | 2.37                   | Doyon, Y et al., Molecular and cellular biology (2004). |
| PAF1      | 2.77                  | 2.66                   | Jaenicke, L.A. et al., Mol Cell (2016). |
| PLK1      | 2.34                  | 2.96                   | Popov, N. et al., Nature cell biology (2010). |
| CCNT1     | 2.48                  |                        | Eberhardy, S.R. & Farnham, P.J., J Biol Chem (2002). |
| KAT5      | 2.41                  | 2.40                   | Frank, S.R. et al., EMBO Rep (2003). |
| AURKA     | 2.25                  |                        | Otto, T. et al., Cancer Cell (2009). |
| RUVBL2    | 1.46                  | 1.50                   | Wood, M.A. et al., Mol Cell (2000). |
| RUVBL1    | 1.30                  | 1.53                   | Wood, M.A. et al., Mol Cell (2000). |

| Gene name | Unique peptide counts |
|-----------|-----------------------|
| MYCN      | 83                    |
| TRRAP     | 54                    |
| GTFIIIC1  | 39                    |
| HCFC1     | 15                    |
| GTFIIIC3  | 13                    |
| GTFIIIC4  | 8                     |
| GTFIIIC5  | 8                     |
| GTFIIIC2  | 7                     |
| TOP2A     | 4                     |

**C**

| Gene name | Unique peptide counts |
|-----------|-----------------------|
| MYC       | 30                    |
| TRRAP     | 109                   |
| GTFIIIC1  | 16                    |
| GTFIIIC3  | 10                    |
| GTFIIIC5  | 8                     |
| GTFIIIC2  | 5                     |
| TOP2A     | 23                    |

**D**

**E**

**F**
Figure S1: Related to Figure 1.

A. Table of previously identified protein-protein interactions of MYC and N-MYC proteins that were confirmed by our analysis. The table shows normalized log$_2$ ratios of peptides purified from SH-EP cells expressing HA-tagged N-MYC$_{\text{wt}}$ (wt) or N-MYC$_{\text{mut}}$ (mut) relative to empty control vector (Ctr).

B. Immunoblots of α-HA immunoprecipitates from SH-EP cells expressing HA-tagged N-MYC$_{\text{wt}}$ or N-MYC$_{\text{mut}}$ or an empty control vector (Ctr). The input corresponds to 1% of the amount used for precipitation.

C. Table of mass spectrometry results of N-MYC immunoprecipitates from MYCN-amplified Kelly cells. Shown are peptide numbers of the indicated proteins.

D. Table of mass spectrometry results of MYC immunoprecipitates from U2OS cells expressing HA-tagged MYC. Shown are peptide numbers of the indicated proteins.

E. Immunoblots of α-TF3C5 immunoprecipitates from IMR-5 cells. The input corresponds to 1% of the amount used for precipitation. Non-specific IgG was used for control immunoprecipitation.

F. Peptide arrays showing binding of Aurora-A to peptide microarrays of N-MYC and MYC. 23mer peptides were spotted, each shifted by 5 amino acids. X-axis shows the position of peptide midpoints and the y-axis shows relative binding. Positions of conserved domains (“MYCboxes”) are indicated at the top of the graph. Data are shown for two concentrations of Aurora-A.
Figure S2 Büchel et al.

A

Normalized tags

5kb

12
10
8
6
4
2
0

N-MYC to TFIIIC5 [bp]

input

TFIIIC5

input

RAD21

CTCF

CTCF motif

B-box

E-box

GCLM

MLLT1

4kb

70
60
50
40
30
20
10
0

Normalized tags

input

N-MYC

TFIIIC5

input

RAD21

CTCF

CTCF motif

B-box

E-box

B

Frequency

0
400
800
1,200
1,600
2,000

Absolute distance

N-MYC to TFIIIC5 [bp]

D

TFIIIC5

5,220

2,053

13,350

N-MYC

Pol II promoter

1.3e-817

2.8e-10

5.4e-81

2.0e-186

Input

Pol III promoter

8.6e-1427

1.0e-3

2.3e-193

2.3e-193

intergenic sites

1.3e-10

1.2e-212

6.0e-283

1.9e-207

E

q-value=8.5e-9

Identified motif

F

E-value

1.3e-10

9.0e-159

6.0e0

Relative motif frequency [x10^-4]

intergenic sites

G

N-MYC/TFIIIC5

1,117

936

22,543

CTCF

p<1e-6
Figure S2: Related to Figure 2.

A. Genome browser tracks at the GCLM and MLLT1 loci illustrating DNA-binding of the indicated proteins. ChIP sequencing was performed on MYCN-amplified IMR-5 cells. The positions of B- and E-boxes and of CTCF motifs are indicated by vertical lines. Upper input is for ChIP sequencing of N-MYC and TFIIIC5, lower input for ChIP sequencing of RAD21 and CTCF.

B. Histogram demonstrating absolute distance between N-MYC and TFIIIC5 peak summits of N-MYC/TFIIIC5 joint peaks (n=2,053). The histogram is plotted at a resolution of 20 bp.

C. Tag density distribution of N-MYC and TFIIIC5 at overlapping sites around the transcription start site (TSS) of genes transcribed by Pol II.

D. De novo motif search in N-MYC- and/or TFIIIC5-bound regions. E-values were calculated using a binominal test and normalized to the number motifs in the database and are shown only if the respective motif was enriched in the bound sequences.

E. Sequence and q-value of the de novo identified motif in overlapping N-MYC/TFIIIC sites and comparison to a published CTCF motif (JASPAR MA0139.1).

F. Central enrichment of E-box, CTCF and AP2a (as negative control) motifs in the N-MYC peak of N-MYC/TFIIIC5 joint sites in intergenic sites. The E-value is calculated by a binominal test and adjustment for the number of motifs tested.

G. Venn diagram documenting genome-wide overlap of N-MYC/TFIIIC5 joint binding sites with CTCF. The p-value was calculated using a permutation test with 100,000 iterations.
Figure S3 Büchel et al.

A

B

RAD21

C

TFIIIC5

D

N-MYC

E

RAD21

Input [%]

TFIIIC1
TFIIIC2
TFIIIC5
CDK2

Input [%]

CDK14
intergenic (Chr 5)
CLINT1 LHFPLMEI4
negative region

Input [%]

CDK14
intergenic (Chr 5)
intergenic (Chr 3)
intergenic (Chr 14)
intergenic (Chr 6)
intergenic (Chr 21)
intergenic (Chr 16)
ERCC

Input [%]

BIRC5
PPRC1
RCC1
GALNT14
EIF2B5
EIF4H
PLK1
NME1
MEL1
ERCC
IgG

Input [%]

BIRC5
PPRC1
RCC1
GALNT14
EIF2B5
EIF4H
PLK1
NME1
MEL1
ERCC
IgG

Input [%]

BIRC5
PPRC1
RCC1
GALNT14
EIF2B5
EIF4H
PLK1
NME1
MEL1
ERCC
IgG
Figure S3: Related to Figure 3.

A. Immunoblot showing levels of the indicated proteins in response to depletion of TFIIIC2 or TFIIIC5. IMR-5 cells expressing an inducible shRNA directed against TFIIIC2 or TFIIIC5 were treated with doxycycline (Dox) for 48 hr or with ethanol (EtOH) as control.

B. ChIP experiment documenting binding of RAD21 to the indicated loci upon depletion of TFIIIC5. Error bars show SD of technical triplicates. These binding sites contain no detectable TFIIIC5 or N-MYC peak.

C. ChIP experiment showing binding of TFIIIC5 to tRNA genes upon depletion of TFIIIC5. Error bars show SD of technical triplicates from one experiment (n = 2).

D. ChIP experiment documenting binding of N-MYC and RAD21 to the indicated loci upon depletion of TFIIIC2. Error bars show SD of technical triplicates from one representative experiment (n = 3).

E. ChIP experiment documenting binding of RAD21 to the indicated loci upon depletion of N-MYC. Error bars show SD of technical triplicates. These binding sites contain no detectable TFIIIC5 or N-MYC peak.
Figure S4 Büchel et al.

A) shTFIIIC5 expression in IMR-5 and SH-EP cells treated with EtOH or DOX.

B) Relative tRNA expression levels in 48 and 96 h for tRNA-Tyr and tRNA-Leu after treatment with EtOH or Dox.

C) Cell morphology after treatment with gRNAs #1 to #8.

D) Cell cycle distribution after treatment:

| Treatment | G1 (%) | S (%) | G2/M (%) |
|-----------|--------|-------|----------|
| EtOH      | 53.3   | 35.2  | 10.3     |
| Dox       | 47.9   | 36.1  | 14.2     |
Figure S4: Related to Figure 4.

A. Clonogenic assay of IMR-5 and SH-EP neuroblastoma cells after shRNA-mediated knockdown of TFIIIC5. Colonies were stained with crystal violet.

B. Expression of indicated tRNAs in IMR-5 cells after shRNA-mediated depletion of TFIIIC5 or a non-targeting control (NTC). Doxycycline or ethanol was added for 48 hr or 96 hr as indicated. Error bars show SD of technical triplicates.

C. Clonogenic assay of IMR-5 cells after sgRNA-mediated knockout of TFIIIC5. Colonies were stained with crystal violet. Multiple independent gRNA sequences were used.

D. Cell cycle distribution of IMR-5 cells after shRNA-mediated depletion of TFIIIC5. Data are taken from a propidium-iodide stained FACS experiment of cells before and 48 hr after addition of doxycycline.
Figure S5: Related to Figure 5.

A. Negative controls for PLAs from SH-EP cells. The indicated antibodies were added to SH-EP cells that do not express N-MYC. Nuclei are stained using Hoechst.

B. Negative controls for PLAs from IMR-5 and SH-EP cells. The panels show PLA assays in which cells were incubated with secondary antibody only. Nuclei are stained using Hoechst.

C. Cell cycle distribution as determined by PI-FACS after release of IMR-5 cells from a double-thymidine block for the indicated times (n = 5). Left axis refers to viable cells in G1 phase shown in blue. Right axis refers to viable cells in S phase shown in red as well as G2/M phase shown in grey.

D. Cell cycle distribution as determined by PI-FACS after re-stimulation of serum-starved IMR-5 cells with 10% FCS (n = 5). Coloring is as in panel C.

E. Immunoblots documenting the levels of the indicated proteins re-stimulation of serum-starved IMR-5 cells with 10% FCS (n = 4).

F. Quantification of the PLA assay between N-MYC and Aurora-A after re-stimulation of serum-starved cells. The percentage of cells in S phase from one representative experiment is indicated in parallel. Error bars show SD of technical triplicates (n = 3).

G. The panels show representative pictures from PLAs documenting complex formation between N-MYC and the indicated proteins (n = 2). Nuclei are stained using Hoechst. Red dots show PLA signals resulting from interactions of N-MYC and the indicated proteins.

H. PLA assays documenting complex formation between N-MYC and TFIIIC5 after release from a double-thymidine block (n = 3). Nuclei are stained using Hoechst. Red dots show PLA signal for interaction of N-MYC and TFIIIC5.
Figure S6 Büchel et al.

A

Protein levels upon CD532 treatment

CD532/DMSO [%]

0 20 40 60 80 100 120

Time [h]

N-MYC

Aurora-A

DMSO

CD532

DMSO

CD532

DMSO

CD532

DMSO

CD532

Figure S6 Büchel et al.

B

EiOH  MG-132

DMSO  CD532

DMSO  CD532

DMSO  CD532

DMSO  CD532

DMSO  CD532

DMSO  CD532

Figure S6 Büchel et al.

C

Kernel density

N-MYC occupancy

[log2 reads]

0 2 4 6 8 10 12

D

Occupancy CD532/DMSO

[log2FC]

N-MYC

TFIIIC5

p=6.0e-81  p=1.9e-126

Figure S6 Büchel et al.

E

N-MYC  9,442  7,994  27,742  TFIIIC5

p<1e-6

163

in tRNA promoters

7,994

N-MYC/TFIIIC5 binding sites

3,946

in Pol II promoters

3,885

inter-/intragenic

Figure S6 Büchel et al.

F

Mean tag density [tags/50bp]

Distance to TFIIIC5 peak [kb]

Figure S6 Büchel et al.
Figure S6: Related to Figure 6.

A. Immunoblots (left) and quantification (right) documenting decrease in overall N-MYC and Aurora-A levels after exposure of IMR-5 cells to CD532 (1 µM) for the indicated times. Error bars show SD (n = 3).

B. Immunoblot documenting levels of Aurora-A and N-MYC after 4 hr exposure to 1 µM of CD532 in the presence of the proteasome inhibitor MG-132 or ethanol (EtOH) as control (n = 2).

C. Change in N-MYC occupancy upon CD532 treatment of all N-MYC peaks (n=15,403). Reads were counted in a window of 250 bp around the peak summit, log$_2$-transformed and the probability density estimation was calculated using a Gaussian kernel function with a bandwidth of 0.1.

D. Box plots documenting changes in N-MYC and TFIIIC5 occupancy at overlapping binding sites in Pol II promoters after exposure of non-synchronized IMR-5 cells to CD532 (4 hr; 1 µM).

E. Venn diagram documenting genome-wide overlap of N-MYC and TFIIIC5 in IMR-5 neuroblastoma cells after exposure to CD532. The p-value was calculated using a permutation test with 100,000 iterations (top). Diagram showing distribution of joint N-MYC/TFIIIC5 binding sites (bottom).

F. Mean tag density around 28,671 TFIIIC5 binding sites without an overlapping N-MYC peak. Tags were counted in a window of +/-1.5 kb around the TFIIIC5 peak summit at a resolution of 50 bp.
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A) 1kb and 2kb normalized tags for ODC1 and NPM1 genes under different conditions.

B) Pol II-bound genes [%] under different conditions.

C) Reads million mapped reads for different conditions.

D) Pol II occupancy in gene body [counts/kb] and Pol II occupancy at TSS [counts/kb] under different conditions.

E) S phase-arrested +CD532 expression [log2CPM] under different conditions.

F) BrdU readouts for different conditions.

G) Count Blots for different conditions.
Figure S7: Related to Figure 7.

A. Browser plots showing Pol II occupancy at the indicated loci in SHEP-N-MYCER cells expressing a doxycycline-inducible shRNA targeting TFIIIC5. Doxycycline (1 μg ml⁻¹) was added for 30 hr where indicated, EtOH was used as control. The top two traces show N-MYC induced changes (Δ) in Pol II occupancy in control and in TFIIIC5-depleted cells.

B. Empirical distribution function (ECDF) plot showing changes in Pol II traveling ratio under the indicated conditions.

C. Metagene plot of all expressed genes (n=14,650) illustrating distribution of hypo-phosphorylated Pol II (8WG16) within transcribed regions before and five hours after activation of N-MYCER.

D. Occupancy of Pol II in the gene body (top) and at the TSS (bottom) sorted according to gene expression in IMR-5 cells synchronized in S phase and treated with CD532 where indicated. Only expressed genes were used (n=14,927) and 30 equal-sized bins (each representing 500 genes) were calculated using the arithmetic mean.

E. 2D Kernel density blot showing Pol II traveling ratio in S phase arrested cells after two hours of CD532 treatment before and after four hours of N-MYCER-activation.

F. Effect of Aurora-A inhibitors on phosphorylation of the indicated proteins in IMR-5 cells synchronized in S phase. Inhibitors were added for 2 hours in indicated concentrations (n = 4).

G. FACS analysis documenting altered DNA synthesis upon incubation of IMR-5 cells with MK- 5108, CD532 and MLN8237. Upper panels show BrdU/propidium iodide double staining (with BrdU-positive cells marked in green), lower panels show propidium iodide.