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MYC interacts with the human STAGA coactivator complex via multivalent contacts with the GCN5 and TRRAP subunits

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ABSTRACT

MYC is an oncogenic DNA-binding transcription activator of many genes and is often upregulated in human cancers. MYC has an N-terminal transcription activation domain (TAD) that is also required for cell transformation. Various MYC TAD-interacting coactivators have been identified, including the transcription/transformation-associated protein (TRRAP), a subunit of different histone acetyltransferase (HAT) complexes such as the human “SPT3–TAF9–GCN5 Acetyltransferase” (STAGA) complex involved in MYC transactivation of the *TERT* gene. However, it remains unclear whether TRRAP and/or other subunits are directly contacted by MYC within these macromolecular complexes. Here, we characterize the interactions of MYC TAD with the STAGA complex. By protein crosslinking we identify both TRRAP and the GCN5 acetyltransferase as MYC TAD-interacting subunits within native STAGA. We show that purified GCN5 binds to an N-terminal sub-domain of MYC TAD (residues 21–108) and that the interaction of GCN5 and STAGA with this sub-domain is dependent on two related sequence motifs: M2 within the conserved MYC homology box I (MBI), and M3 located between residues 100–106. Interestingly, specific substitutions within the M2/3 motifs that only moderately reduce the intracellular MYC–STAGA interaction and do not influence dimerization of MYC with its DNA-binding partner MAX, strongly inhibit MYC acetylation by GCN5 and reduce MYC binding and transactivation of the GCN5-dependent *TERT* promoter in vivo. Hence, we propose that MYC associates with STAGA through extended interactions of the TAD with both TRRAP and GCN5 and that the TAD–GCN5 interaction is important for MYC acetylation and MYC binding to certain chromatin loci.

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1. Introduction

The MYC oncoprotein controls a wide variety of cell biological processes in metazoans, including cell growth, cell cycle, cell proliferation, differentiation, and apoptosis [1]. MYC expression is highly regulated in normal cells and its uncontrolled overexpression is oncogenic in vertebrate animals and associated with most types of cancers in humans. Hence, there is much interest in MYC as a potential drug target for cancer treatment [2–4]. MYC functions as a DNA-binding transcription regulator [5,6], which modulates transcription of many, if not most, genes transcribed by all three nuclear RNA polymerases [7,8], and references therein). MYC has a C-terminal basic Helix–Loop–Helix leucine–Zipper (bHLHZ) domain that is required for heterodimer formation with its

obligatory bHLHZ partner MAX and for binding to E-box DNA elements having the consensus sequence CA(C/T)GTG [1]. The N-terminus of MYC (residues 1–263) contains a transcription activation domain (TAD) and includes the so-called MYC homology boxes (MBI, MBII, and MBIII), which are amino acid sequences conserved between MYC and its family members MYCN and MYCL [5]. Both the bHLHZ and TAD domains are required for most of the biological functions of MYC, including its neoplastic cell transformation activity [1,5,6]. While the TAD of MYC has also been implicated in transcription repression, most genes are transcriptionally activated by MYC [5–8]. How MYC activates transcription is, however, still poorly understood. A variety of proteins that interact with the TAD of MYC have been identified but only few have been shown in independent studies to be recruited by MYC and to mediate the transactivating functions of MYC on target promoters. Among these, the transactivation/transformation-associated protein (TRRAP) and several histone acetyltransferases (HATs) that stably associate with TRRAP into large multiprotein complexes (i.e., TRRAP–HAT complexes below), and the positive transcription elongation factor b (P-TEFb) have been most studied and have emerged as important cofactors for MYC-dependent transcription in mammalian cells [5,6]. In different contexts TRRAP–HAT complexes were shown either to mediate MYC induction of nucleosomal histones H3 and H4 hyperacetylation [9–12]

Abbreviations: ATAC, ADA-Two-A Containing; bHLHZ, basic Helix–Loop–Helix leucine–Zipper; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; HAT, histone acetyltransferase; MB, MYC homology box; RNAi, RNA interference; RT-qPCR, reverse transcription quantitative/real-time polymerase chain reaction; STAGA, Spt-Ada-Gcn5 Acetyltransferase; STAGA, SPT3–TAF9–GCN5/PCAF Acetyltransferase; TAD, transcription activation domain

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or to facilitate MYC-dependent recruitment of Mediator on target promoters [13,14]. On the other hand, P-TEFb contributes to MYC-induced release of paused RNA polymerase II [15–18], a general transcription elongation function of MYC that was reported to amplify transcription of all active genes in proliferating cells [7,8]. Thus, MYC can activate transcription via either TRRAP-dependent or TRRAP-independent pathways and the contributions of each pathway are cell type/context-dependent [11].

TRRAP was originally identified as a MYC TAD-interacting protein required for MYC-dependent transformation of rodent fibroblasts and mapping analyses indicated that MYC TAD residues N-terminal to MBI (i.e. residues 20–32 and 39–48) and the MBII region (residues 129–145) are essential for the intracellular MYC–TRRAP interaction and for cell transformation [19]. Although MYCN (and probably MYC itself) interacts directly with TRRAP [20], it has remained unclear whether TRRAP contacts directly both the MBI and MBII regions. TRRAP (or Tra1 in yeast) was independently identified as a subunit of several different multiprotein TRRAP–HAT complexes having transcription co-activator functions. These include the yeast NuA4 complex [21] and the homologous mammalian TIP60 complex [22], which preferentially acetylate histone H4, and the yeast SAGA (Spt–Ada–Gcn5 Acetyltransferase) and derivative SAGA-like (i.e., SLIK/SALSA) complexes [23,24] and their homologous mammalian STAGA (SPT3–TAF9–GCN5/PCAF Acetyltransferase) complexes [25–28], which contain the GCN5 HAT (or its tissue-specific paralog PCAF) and preferentially acetylate histone H3 (reviewed in [29]). It is important to note that GCN5, whose expression is stimulated by MYC to affect global acetylation of chromatin [30] and is important for MYC-mediated cell transformation [31], is also part of a metazoan-specific complex called ATAC (ADA-Two-A Containing), which does not contain TRRAP [32–34]. It is unknown, however, whether ATAC contributes to any MYC function. On the other hand, TRRAP was also found in a MYC regulatory complex, the mammalian p400 complex, which is related to the TIP60 complex but lacks HAT activity [35]. Such promiscuity of TRRAP and associated cofactors has made it difficult to ascertain which TRRAP complex is directly recruited by MYC and mediates MYC transactivation on physiological target promoters. However, a specific interaction of the human STAGA complex with MYC was demonstrated, which required TRRAP and the TAD 1–110 region of MYC [36]. A similar direct interaction of MYC with the yeast SAGA complex was also reported [37]. Moreover, a role of human STAGA in MYC transactivation of the telomerase reverse transcriptase (*TERT*) gene in cancer cell lines was established unambiguously [14]. Indeed, knockdown of the core STAGA-specific subunit STAF65γ (hSPT7) disrupted the complex and prevented MYC-dependent recruitment to the *TERT* promoter of the SPT3–TAF9 module, and inhibited Mediator recruitment concomitant with a reduction in *TERT* transcription. However, knockdown of STAF65γ did not affect MYC-dependent recruitment of other STAGA components to the *TERT* promoter, including TRRAP and GCN5; this suggested the possibility that MYC might directly interact with TRRAP and GCN5 to recruit native STAGA complexes to chromatin [14]. Notably, the cytoplasmic MYC–nick cleavage product of MYC, which contains the N-terminal TAD (1–298) but lacks the C-terminal bHLHZ domain, has been shown to promote alpha-tubulin acetylation and cell differentiation via recruitment of TRRAP and GCN5, suggesting the possibility that STAGA might also functionally interact with the TAD sequences of MYC–nick in the cytoplasm [38].

Early observations in yeast suggested that activators recruit the NuA4 and SAGA coactivator complexes by contacting only the essential Tra1/TRRAP subunit common to these complexes [39]. However, other crosslinking analyses both with purified HAT complexes and promoter-bound transcription complexes showed that several acidic activators recruit the yeast SAGA complex by contacting not only Tra1/TRRAP but also the Ada1, Taf6 and Taf12 subunits [40,41]. In mammalian cells, the direct interacting targets of activators within the native TRRAP–HAT complexes TIP60 and STAGA remain generally unknown, although it is often assumed that TRRAP is the common

and sole target. To our knowledge only one study has directly addressed this issue and showed by using a crosslinking analysis that the interaction of the p53 tumor suppressor protein with the STAGA complex involves multivalent contacts of different regions of p53 with the GCN5, TAF9, and ADA2B subunits but, surprisingly, not with TRRAP [42]; hence, the previously reported direct interaction of p53 with isolated TRRAP [43] could be relevant for p53 recruitment of other TRRAP complexes such as the TIP60 complex. Similarly, it is generally assumed that MYC recruits GCN5 as part of the STAGA complex via direct contacts of its TAD domain with only the TRRAP subunit [5,6,31,36]. However, this has never been verified and it has remained possible that MYC could contact other subunits within STAGA.

Here we have further analyzed the physical and functional interactions of STAGA with the TAD of MYC. We show that both the TRRAP and GCN5 subunits within native STAGA complexes crosslink to MYC TAD (residues 1–263) and that GCN5 directly binds a TAD sub-domain (21–108) that contains MBI but lacks MBII. Within this TAD sub-domain two related sequence motifs M2 (at the core of MBI) and M3 (residues 100–106) are important for the binding of GCN5 and the STAGA complex. Notably, single amino acid substitutions within the M2/3 motifs, which moderately affect the intracellular MYC–STAGA interaction, strongly inhibit GCN5-mediated acetylation of MYC in cultured cells. Moreover, the M2/3 motifs are important for MYC binding and transactivation of the human *TERT* gene promoter in human cells. Hence, our results suggest that MYC interacts with the STAGA complex via multivalent contacts with both the TRRAP and GCN5 subunits and that the GCN5 interaction with the TAD does not merely facilitate STAGA recruitment but also regulates acetylation of MYC at distant domains and MYC binding to specific chromatin loci.

2. Materials and methods

2.1. DNA plasmids

The human *TERT* promoter-luciferase reporter vector (p2xEB) and pcDNA–mGCN5 expression vector were described elsewhere [36]. The pSG5–hGCN5–S expression vectors for human GCN5–S (short form, 1–476) and deletion mutants (1–388; 111–476; 252–476; 1–110; 111–251; 252–388; 389–476) were received from Saadi Khochbin and described previously [44]. The bacterial expression vector for human GCN5–S, pRSETA–His6–GCN5–S, was obtained from Richard A. Currie and the expression vectors for glutathione S-transferase (GST) fused to human c-MYC TAD – i.e., GST–MYC(1–263), and most deletion mutants – were obtained from Bruno Amati [45]. The expression vectors for GST–MYC(2–108), GST–MYC(21–108), GST–MYC(47–108) and GST–MYC(53–108) were generated by inserting between the Bam HI and Eco RI sites of pGEX–2T corresponding Bam HI–Eco RI fragments of human MYC cDNA generated by PCR (with a stop codon after position 108). The GST–MYC(2–108) mutants: D26A, E54A, E100A, E[54,100]A, and D26A–E[54,100]A were created by site-directed mutagenesis of GST–MYC(2–108) using the QuickChange site-directed mutagenesis kit (Stratagene). The bacterial expression vector pRSET–His6–MycE [54,100]A mutant was obtained by site-directed mutagenesis of pRSET–His6–Myc [46]. The mammalian expression vectors for human c-Myc, pCbs–Flag–c-Myc WT or E[54,100]A mutant, were obtained by replacing the Xma I–Hind III fragment of pCbs–Flag–MaxK[144,145]R [47] with the Xma I–Hind III fragment obtained by PCR of either pRSET–His6–Myc or pRSET–His6–MycE[54,100]A using the Xma I-containing primer 5′-GGTCCCCCGGATGCCCTCAACGTTAGCTTC-3′ and the Hind III-containing primer 5′-CTGCCAAGCTTACGCACAAGA GTTCCG-3′. The pSUPER–Tet.retro.puro plasmid was obtained by replacing the H1 promoter-containing Eco RI–Bgl II fragment of pSUPER.retro.puro (OligoEngine) with an Eco RI–Bgl II fragment containing a modified H1 promoter with one tetracycline operator 2 (TetO2) downstream of the TATA box. The pSUPER–Tet–GCN5–

2031 vector was obtained by cloning the human GCN5-si2031 annealed oligonucleotides: 5'-GATCCCGCTGATTGAGCGCAAAGTGTCAAGAGACTGTTTGGCTCAATCAGCTTTTA-3' (sense) and 5'-AGCTTAAAGAGCTGATTGAGCGCAAAGTGTCTCTTGAACAGTTTGGCTCAATCAGCGG-3' (antisense) between the Bgl II and Hind III sites of pSUPER-Tet. retro. puro plasmid. The specific targeting sequences are underlined. pSUPER-GL2 was described previously [45]. All plasmid constructs were verified by DNA sequencing.

2.2. Cell culture, transfection, and luciferase assay

HeLa and HEK293 cell lines and IMR90 normal human lung fibroblasts were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO₂. HeLa and IMR90 cells were transfected with the indicated expression and reporter plasmids using Lipofectamine2000 (Invitrogen) and HEK293 cells using ExpressFect transfection reagent (Denville), according to the manufacturer's instructions. Corresponding empty vectors were used to keep total DNA constant. For luciferase reporter assays, HeLa cells were transfected in 10-cm plates with 7 µg of either an empty pCbs vector or pCbs-Flag-Myc WT or E[54,100]A mutant, and 2.5 µg of p2xEB (hTERT-Luc) and 0.5 µg of pCMV-β-galactosidase. Luciferase assays were performed as previously described [36]. Luciferase activities were the mean ± standard deviation (SD) from at least three independent transfection experiments, each performed in duplicate. Significant differences in luciferase assays and in all other quantitative assays below were $p < 0.05$ by Student's t-test.

2.3. Immunoprecipitation and Western blotting

Cell lysis, immunoprecipitations and Western blotting were performed essentially as described previously [45], with the following antibodies: SPT3 [25]; YEATS2 [34]; STAF65γ, TBP, TAF9, TAF12, MED1 (obtained from Robert G. Roeder); FAM48A (gift from Jiahui Han); Actin (I-19), CDK9 (H-169), GCN5 (N-18), MAX (C-17), MYC (N-262), TRRAP (T-17) (Santa Cruz Biotechnology); Acetyl-Lysine (Acetyl-K, Cell Signaling Technology); Flag M2, Anti-Flag M2 Affinity gel, and Vinculin (Sigma). The X-ray films were scanned with an HP precisionscan Pro 3.1 scanner and densitometry analyses were performed with NIH ImageJ software.

2.4. GST pull-down and protein crosslinking assays

Glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli*, and standard GST pull-down assays were performed essentially as previously described [28] with either HeLa nuclear extracts or recombinant GCN5-S (short isoform) that was expressed in *E. coli* and purified as reported [45]. Alternatively, GST pull-down assays were performed with [³⁵S]methionine-labeled recombinant GCN5 proteins, which were obtained by in vitro transcription and translation of pSG5-hGCN5-S (WT or mutants) vectors using the TNT system (Promega), according to the manufacturer's instructions; radio-labeled proteins on SDS-PAGE gels were detected by fluorography. Dual GST pull-down/crosslinking assays were performed similarly to a previous report [42] by incubating 200 µl of HeLa nuclear extract, or 40 µl of purified STAGA complex [28], with 10 µg of immobilized GST or GST-MYC(1–263) fusion proteins for 4 h at 4 °C in BC160 buffer (20 mM Tris-HCl [pH 7.9], 160 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% IGEPAL CA-630, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). The resin was washed three times with cold HEPES-buffered BC200 (20 mM HEPES [pH 7.9], 200 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% IGEPAL CA-630, 10 mM 2-mercaptoethanol, 0.2 mM PMSF) and twice with crosslinking buffer (20 mM HEPES [pH 7.9], 100 mM KCl). The resin was then resuspended in 200 µl of crosslinking buffer containing variable concentrations (5–80 µM) of the crosslinking agent dithiobis[succinimidylpropionate] (DSP; Thermo

Scientific Pierce) and incubated 20 min at room temperature with occasional mixing. The crosslinking reaction was quenched by adding 1 M Tris-HCl (pH 7.5) to 50 mM final concentration and incubating for 20 min at room temperature. Uncrosslinked proteins were removed by washing the resin three times (10 min each) with urea wash buffer (30 mM Tris-HCl [pH 7.5], 100 mM KCl, 0.1% IGEPAL CA-630, 8 M urea) and twice with BC100 buffer (same as BC160 but with 100 mM KCl). Crosslinks were reversed and proteins were eluted from the beads by heating at 95 °C for 5 min in SDS-PAGE sample loading buffer containing 0.1 M dithiothreitol (DTT). Alternatively, for crosslinking reactions with purified STAGA, the resin was incubated with elution buffer (20 mM Tris-HCl [pH 7.9], 100 mM NaCl, 0.2% Sarkosyl, 0.1 M DTT) at 37 °C for 30 min. Eluted proteins were resolved by SDS-PAGE and analyzed by Western blot with the indicated antibodies. Crosslinking of live cells with DSP was performed essentially as recommended by the manufacturer (Pierce). Cell lysis and immunoprecipitation of Flag-MYC from uncrosslinked and crosslinked cells was performed under non-denaturing conditions, as described previously [45], or with denaturing RIPA buffer (50 mM Tris, [pH 8.0], 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS), as indicated (– or + SDS/DOC).

2.5. Analysis of MYC acetylation in cultured cells

HeLa cells were transfected in 10-cm plates with 7.5 µg of either empty pCbs vector, pCbs-Flag-Myc WT or E[54,100]A mutant. After 48 h, whole cell extracts were prepared and MYC proteins were immunoprecipitated with anti-Flag M2 agarose and analyzed by SDS-PAGE and Western blotting with the Acetyl-K antibody, essentially as described previously [45]. HEK293 cells were transfected with 7.5 µg of either pCbs-Flag-Myc WT or E[54,100]A mutant, and 10 µg pCDNA-mGCN5. After 48 h, the cells were incubated with histone deacetylase (HDAC) inhibitors (20 mM nicotinamide, 4 µM trichostatin A) for 2 h prior to cell lysis, and cell lysates were analyzed by Western blotting.

2.6. RNA interference (RNAi), quantitative reverse transcription-PCR (RT-qPCR) and ChIP assay

For constitutive knockdown of GCN5 by RNAi, HeLa cell lines stably expressing the shRNAs for GCN5 or luciferase (GL2) were obtained by stable transfection with, respectively, pSUPER-Tet-GCN5-2031 or pSUPER-GL2, as previously described [45]. For transient RNAi-mediated knockdown of GCN5, HeLa cells were transfected in 6-cm plates with 5 µg of pSUPER-Tet-GCN5-2031 or pSUPER-GL2 using Lipofectamine2000 and once again 24 h later. On the next day, the medium was changed with puromycin-containing medium (1 µg/ml), and after 24 h, whole cell extracts were prepared for Western blot analyses; total RNA was also purified from a fraction of the cell population for RT-qPCR, as described previously [45]. IMR90 cells were transfected in 10-cm plates at 80–90% confluence with 7.5 µg of pCbs, pCbs-Flag-Myc WT or E[54,100]A mutant using Lipofectamine2000. After 24 h, the cells were transfected again. On the next day, whole cell extracts were prepared and total RNA was purified as above. For RT-qPCR, cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) and quantitated in triplicates by using a MiniOpticon Real-Time PCR system and iQ™ SYBR Green Supermix (Bio-Rad), according to manufacturer's instructions. The following qPCR primers were used: *TERT*, 5'-TCCACTCCCCACATAGGAATAGTC-3' (forward) and 5'-TCCTTCTCAGGGTCTCCACCT-3' (reverse); *MYC*, 5'-GCCACGTCTCCACATCAG-3' (forward) and 5'-TGGTGCATTTTCGGTGTG-3' (reverse). The *ACTB* (β-actin) primers were described previously [43]. Relative mRNA expression was obtained by the Pfaffl method and presented as the mean (± SD) of at least three independent experiments.

For ChIP analyses, HeLa cells transiently transfected with Flag-Myc WT or E[54,100]A mutant were crosslinked in 10-cm plates at 80–90%

confluence with 1% formaldehyde. Cell lysis and shearing of chromatin by sonication were as previously described [9]. The normalized and pre-cleared chromatin samples were immunoprecipitated with 7.5 μ l of FLAG M2 affinity resin at 4 °C overnight. ChIP assays were performed essentially as described previously [45], with the exception that immunoprecipitated chromatin was analyzed in triplicates by real-time qPCR (see above) with human *TERT* promoter-specific primers [48] and with primers to an unrelated non-coding region on chromosome 6 that is not transcribed by Pol II and served as a background control for normalization of immunoprecipitated chromatin [49].

3. Results and discussion

3.1. The N-terminal transcription activation domain (TAD) of MYC interacts with the STAGA complex but not the ATAC complex

We previously demonstrated that MYC interacts with the STAGA co-activator complex *in vitro* and in human cell lines, and that the N-terminal 1–110 amino acid residues of MYC transcription activation domain (TAD) are essential for this interaction [36]. More recently, we and others identified a second human GCN5 complex – i.e., ATAC – that shares several subunits with STAGA, but in most part differs in composition and functions [34,49,50]. Immunoprecipitation experiments of Flag-tagged MYC expressed in human HEK293 cells confirmed the interaction of STAGA subunits with MYC and identified the ATAC-specific subunit YEATS2 as a possible MYC-interacting protein (Fig. 1A); however, the relative amounts of YEATS2 associated with MYC were very low and other ATAC subunits (UBAP2L and NC2 β) were not detected under the conditions used (data not shown). This suggested that ATAC might only weakly interact with MYC *in vivo*. We further tested whether the TAD (1–263) domain of MYC can bind both complexes *in vitro*. GST pull-down experiments were performed with immobilized GST-MYC(1–263) fusion protein and HeLa nuclear extracts. As expected, STAGA subunits were bound to MYC TAD (Fig. 1B, lane 6). In contrast, several ATAC subunits were not detected in the bound fraction (Fig. 1B, lane 6), even after longer exposures (data not shown). We conclude that MYC TAD (1–263) interacts selectively with the human STAGA-type (i.e., STAGA) but not the ATAC-type complexes. Whether

ATAC can interact with MYC via the C-terminal (264–439) region of MYC (Fig. 1C), remains to be investigated.

3.2. MYC TAD contacts both the GCN5 and TRRAP subunits within the STAGA complex

The TRRAP protein is a subunit of several different complexes, which include STAGA and the TIP60 complexes in mammalian cells, and was originally identified as a protein that binds the TAD (1–262) region of MYC, in a manner that requires MBII and sequences near MBI [19,20]. However, whether MYC TAD indeed contacts TRRAP and/or other subunits within the native STAGA complex has remained unclear. To identify the protein subunits within the STAGA complex that interact with the TAD (1–263) of MYC we used an *in vitro* GST pull-down and protein crosslinking approach with dithiobis(succinimidylpropionate) (DSP) [42]. DSP is a thiol-cleavable homobifunctional chemical crosslinker that covalently links amine groups within a 12 Å distance. Cell nuclear extracts were incubated with immobilized GST-MYC(1–263), and after extensive washes bound proteins were crosslinked with varying concentrations of DSP. After washing away non-crosslinked proteins with a urea-containing buffer, crosslinks were reversed and proteins were eluted with DTT-containing SDS gel loading buffer. Eluted proteins were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against most STAGA subunits. Only TRRAP and GCN5 were found to strongly crosslink to GST-MYC(1–263) (Fig. 2A). Under the conditions used, up to ~1% of total TRRAP and GCN5 present in the extract was crosslinked. Like most other STAGA subunits, the ATAC-specific subunit YEATS2 and the Mediator subunit MED1 did not crosslink to MYC TAD.

To verify that the crosslinking of MYC TAD to TRRAP and GCN5 was due to STAGA, the GST pull-down and crosslinking assays were repeated with STAGA complexes immunopurified from a HeLa cell line expressing Flag-tagged SPT3 [28]. Consistent with the above results, 10–20% of TRRAP and GCN5 within purified STAGA were specifically crosslinked to GST-MYC(1–263) but not to GST alone (Fig. 2B, lane 4 vs. 5). In addition, since DSP is cell-permeable, we found that TRRAP and GCN5 can also be crosslinked to Flag-tagged MYC in transfected HEK293 cells in a DSP-dependent manner (Fig. 2C). Hence, the above crosslinking results suggested that MYC TAD (1–263) binds to the STAGA complex

Fig. 1. MYC TAD specifically interacts with the STAGA complex. (A) Flag-MYC (+) or empty vector (–) was transfected in HEK293 cells, and cell extracts (Input) were immunoprecipitated with the FLAG antibody and analyzed by SDS-PAGE and Western blot with the indicated antibodies. (B) GST pull-down assay. GST or GST fused to MYC-TAD(1–263) (GST-TAD) immobilized on glutathione agarose (Resin) was incubated with HeLa nuclear extracts (Input) and bound and unbound fractions were analyzed by SDS-PAGE and Western blot with the indicated antibodies to STAGA and ATAC subunits. About 1% of STAGA in the input extract bound specifically to MYC TAD. (C) Domain structure of MYC with conserved MYC boxes (MBI, II, and III), nuclear localization signal (NLS) and bHLH domain.

Since we previously demonstrated that MYC TAD directly binds STAGA and that the sub-domain 2–108 is necessary and sufficient [36], we tested as above the possible role of the M2/3 motifs. Deletion of residues 54–56 in M2 ($\Delta 54$ –56) reduced the binding to all STAGA subunits analyzed in cell nuclear extracts and the double E[54,100]A substitution in M2 and M3 motifs had an even stronger inhibitory effect (Fig. 5A and B). Notably, these subtle TAD sequence alterations did not substantially affect the binding of the CDK9 subunit of P-TEFb (Fig. 5B), which was reported to bind to the MBI region residues 30–70 [16]. Interestingly, the TIP48 subunit of the p400 and TIP60 complexes, which interacts with both the TAD 1–108 and MBI regions [36], was also not significantly affected by the double E[54,100]A substitution (Fig. 5B).

Fig. 3. GCN5 directly binds to MYC TAD (2–108) sub-domain and requires TAD residues 54–56 (M2 motif) and 99–108 (M3 motif). (A) GST pull-down assays performed with purified recombinant human GCN5-S and analyzed by SDS-PAGE and Western blot with a specific GCN5 antibody. The purified GST-MYC fusion proteins used are shown in Supplemental Fig. S1. (B) Summary of results of at least two independent GST-pull-down experiments as above. (C) Alignment of homologous amino acid sequences in human MYC family members (MYC, MYCN, MYCL). Numbers indicate amino acid coordinates in MYC. Asterisks above the sequence indicate the aspartic and glutamic acid residues that were changed to alanine in the MYC TAD mutants in panel D (below). Sequences between residues 26 and 47 and between 65 and 98 are not shown. The conserved M1 motif (22–46) is highlighted with dotted lines. The M2 motif (within MBI) and the M3 motif are highlighted in gray. MYCL lacks a homologous M3 motif. Sequences in p53 and Adenovirus 5 E1A proteins that are known to bind GCN5/PCAF are shown and residues matching the M2 or M3 motifs (Consensus M2/3) are highlighted in gray. In the consensus M2/3 sequence ϕ represents a hydrophobic amino acid. The M2 motif of MYC and homologous motif in E1A proteins are flanked by a conserved proline within the PSR/PSH or PGH motif (underlined). (D) Relative binding of recombinant GCN5 to GST-MYC(2–108) and the indicated point mutants in M1, M2 and M3 motifs was analyzed by in vitro pull-down as above, and results (average $\pm$ SD) of at least three independent experiments are shown in relative units (arbitrarily set to 100 for binding to wt).

Fig. 4. MYC TAD interacts with the HAT and ADA2 domains of GCN5. Left, summary of results obtained from at least two independent GST pull-down experiments with GST-MYC(1–204) and in vitro translated recombinant GCN5-S (#1) or the indicated deletion mutants (#2–8). The diagram depicts the positions of the GCN5 HAT domain, ADA2 domain, and bromodomain (BrD) [44]. Right, Fluorogram of SDS-PAGE gel containing 35 S-methionine-labeled GCN5 and mutant proteins from one representative GST pull-down experiment. Input GCN5 proteins and the GST or GST-MYC bound fractions are shown.

Fig. 5. The interaction of STAGA with MYC TAD (2–108) requires the M2 and M3 motifs. GST pull-down experiments were performed with HeLa nuclear extracts (input) and either GST or the indicated GST-MYC (2–108) wild-type (wt) or mutant (Δ 54–56 and E[54,100]A) proteins. The bound fractions were analyzed by SDS-PAGE and Western blot with the indicated antibodies. Ponceau S stain of the blot is shown in panel A. Panel B indicates that ~5% of the input STAGA in the extract bound to TAD wt; ~1% bound to Δ 54–56; and only ~0.2% bound to the E[54,100]A mutant.

Thus, these M2/3 mutations do not have a generalized effect on all proteins interacting with the MYC TAD 2–108 sub-domain, but specifically affect the binding of the STAGA complex.

3.4. Role of MYC M2/3 motifs in MYC–STAGA interaction and MYC acetylation in live cells

To address the role of MYC M2/3 motifs in the intracellular MYC–STAGA interaction, Flag-MYC wild type (WT) or the E[54,100]A mutant was expressed in HEK293 cells and analyzed by immunoprecipitation and Western blotting. While both MYC WT and the E[54,100]A mutant bound MAX and the CDK9 subunit of P-TEFb to similar extents, the E[54,100]A mutant showed reduced binding to GCN5 and to all other STAGA components tested (Fig. 6A, lane 5 vs. 6). The effect of the E[54,100]A substitution was, however, modest when compared to the strong inhibition observed in vitro with the isolated MYC TAD (2–108) sub-domain (Fig. 5). This is likely the result of additional interactions of STAGA with MYC outside the 2–108 region that take place in the context of full-length MYC, such as the binding of TRRAP to MBII [19,20].

To further test the functional relevance of the M2/3 motifs in vivo, we analyzed the role of the E[54,100]A substitution in MYC acetylation by GCN5 in cultured cells. We previously showed that GCN5 and the purified STAGA complex can directly acetylate MYC in vitro [45] and ectopic GCN5 induces MYC acetylation in cultured cells [53]. As shown in Fig. 6B (top panel), immunoprecipitation and Western blot analyses of Flag-MYC WT expressed in HeLa cells confirmed both the interaction of MYC with endogenous GCN5 and acetylation of MYC in these cells (lane 2). In contrast, the E[54,100]A mutant had a diminished interaction with endogenous GCN5 (as expected) and, interestingly, its acetylation levels were strongly reduced (lane 3). To determine whether the E[54,100]A substitutions affected MYC acetylation specifically by GCN5, similar analyses were performed in HEK293 cells. These cells are transformed with Adenovirus DNA and express the E1a oncoprotein, which inhibits endogenous GCN5/PCAF HATs. Hence, efficient acetylation of MYC requires overexpression of ectopic GCN5, as shown in Fig. 6B (bottom panel, lane 1 vs. 2). Consistent with the above results, ectopic GCN5 did not efficiently bind or acetylate the E[54,100]A MYC mutant in these cells (Fig. 6B, bottom panel, lane 4 vs. 5). Altogether, these observations suggest that the binding of GCN5 and native STAGA to MYC involves the conserved M2/3 motifs and is required for MYC acetylation in vivo. The importance of N-terminal TAD sequences for both GCN5/STAGA binding and GCN5-mediated acetylation of MYC lysine residues located near the C-terminus of MYC [53] is intriguing and suggests a regulatory (e.g., structural or allosteric) role of the TAD–GCN5 interactions beyond a simple recruiting function.

3.5. MYC transactivation of the human telomerase reverse transcriptase (TERT) gene requires GCN5 and MYC M2/3 sequence motifs

We previously showed that MYC recruits the STAGA complex to activate transcription of the endogenous *TERT* gene in HeLa cervical cancer cells and that the activation mechanisms are distinct from a simple stimulation of histone acetylation [14]. Indeed, disruption of the STAGA complex by knockdown of the core subunit STAF65 γ inhibited MYC-dependent *TERT* transcription and recruitment of specific STAGA subunits (i.e., SPT3 and TAF9) and Mediator, but did not affect MYC recruitment of TRRAP and GCN5 or acetylation of histones H3 and H4 on the promoter [14]. The latter observation can now be explained by the findings reported here of a direct interaction of MYC TAD with both TRRAP and GCN5 within STAGA. However, it has remained unclear whether GCN5 is required for *TERT* transcription. We now verify this by showing that knockdown of GCN5 reduces *TERT* mRNA levels in HeLa cells (Fig. 7A). To further test the role of MYC M2/3 sequence motifs in de novo transcription activation of the *TERT* gene, Flag-tagged MYC WT or the E[54,100]A mutant was expressed in normal human diploid fibroblasts (IMR90 cells). Indeed, these cells have very low levels of endogenous MYC and a silent *TERT* gene that is activated by ectopic MYC expression, which also dramatically extends their life span [11,54]. We found that the E[54,100]A mutant activated endogenous *TERT* in IMR90 cells to a lesser degree than MYC WT (Fig. 7B). In accord with this, the E[54,100]A mutant had a reduced ability to activate an artificial *TERT* promoter-luciferase reporter gene (Fig. 8A). Interestingly, chromatin immunoprecipitation (ChIP) experiments in HeLa cells transfected with either Flag-tagged MYC WT or the E[54,100]A mutant indicated that the mutant does not bind efficiently to the endogenous *TERT* promoter (Fig. 8B). Note that while our ChIP experiments could not detect the *TERT*-binding activity of the E[54,100]A mutant above background, it is clear from its ability to weakly activate *TERT* promoter-dependent transcription (see above) that this mutant has residual *TERT* promoter/chromatin-binding activity. Although the exact mechanism for the reduced binding of MYC E[54,100]A mutant to *TERT* remains to be determined, it seems unlikely that the defective acetylation of this mutant is involved. Indeed, we have shown previously that an acetylation-defective MYC mutant (R6) that has

Fig. 6. The M2/3 motifs of MYC TAD are required for optimal MYC–STAGA interaction and for GCN5-dependent MYC acetylation in vivo. (A) M2/3 motifs are important for optimal intracellular interaction of MYC with STAGA. Flag-MYC wild type (WT) or the E[54,100]A mutant was immunoprecipitated from transfected HEK293 cells and associated proteins were analyzed by Western blotting. (B) M2/3 motifs are required for GCN5-dependent MYC acetylation. Flag-MYC (WT) or E[54,100]A mutant were transfected in HeLa cells (top) or HEK293 cells (bottom). HEK293 cells were also transfected with GCN5 (+) or an empty vector (–) as indicated. MYC proteins and associated GCN5 were immunoprecipitated with the FLAG antibody and analyzed by Western blot. MYC acetylation was detected with an acetyl-lysine antibody (AcK).

each of the six major acetylated lysine residues changed to arginine can still activate the *TERT* promoter [45]. These results suggest the intriguing possibility that the N-terminal TAD of MYC, and specifically the M2/3 sequence motifs, might influence the functions of the C-

terminal bHLHZ domain in vivo and, hence, that the physical interactions of STAGA (and perhaps other MYC cofactors) with the TAD could potentially regulate MYC binding to specific chromatin loci. Consistent with such a possibility, it has been reported that the

Fig. 7. Human *TERT* gene transcription depends on GCN5 and MYC M2/3 motifs. (A) Knockdown of GCN5 in HeLa cells specifically reduces *TERT* mRNA levels. Cells were transiently transfected with vectors encoding a control shRNA (GL2) or the GCN5 shRNA. Left, Western blot analysis of transfected cell extracts. Right, relative amounts of *TERT* and MYC mRNAs (normalized to *ACTB* mRNAs) were obtained by RT-qPCR analyses in control cells (black bars, set arbitrarily to 1) and in GCN5 shRNA cells (open bars). (B) The M2/3 motifs are required for optimal activation of *TERT* by ectopic MYC in normal human fibroblasts. IMR90 fibroblasts were transfected with Flag-MYC WT or the E[54,100]A mutant, or the corresponding empty vector (–). Left, Western blot of transfected cells. Right, relative *TERT* mRNA levels (normalized to *ACTB* mRNAs) were determined by RT-qPCR. *TERT* mRNA levels in WT-transfected cells were arbitrarily set to 1.0. Results are the mean (± SD) of three independent experiments (* indicates $p < 0.05$).

Fig. 8. The M2/3 motifs influence MYC binding and transactivation of the *TERT* promoter. (A) MYC transactivation of a *TERT* promoter-luciferase reporter is dependent on the integrity of the M2/3 motifs. Top, Western blot of cells transfected with either Flag-MYC WT or E[54,100]A mutant, or empty vector (–). Bottom, relative luciferase activities in transfected cells are relative to WT-transfected cells (arbitrarily set to 1). (B) Binding of MYC to the endogenous *TERT* promoter in HeLa cells is dependent on the integrity of the M2/3 motifs of MYC TAD. Relative binding of Flag-tagged MYC WT or E[54,100]A mutant to the endogenous *TERT* promoter in transfected HeLa cells was determined by ChIP with the FLAG antibody and quantitated by real-time qPCR with *TERT* promoter-specific primers and primers for a control non-coding genomic region used for normalization (binding in empty vector transfected cells was set to 1). Two independent ChIP experiments are shown (black and gray bars) each analyzed in triplicates. Expression of similar amounts of MYC WT and E[54,100]A mutant was verified by Western blot (panel A; and data not shown).

yeast Gal4 activator requires the interaction with Tra1/SAGA for optimal binding to target promoters in vivo [55].

4. Concluding remarks

The results presented here provide a more detailed understanding of the molecular interactions between MYC and its transcription coactivator complex STAGA. Our crosslinking analyses of STAGA bound to MYC TAD (1–263) show for the first time that both GCN5 and TRRAP are in direct physical proximity to MYC TAD. These results and the

demonstration that purified GCN5 directly binds to the MBI-containing sub-domain of MYC suggest a revised model for MYC recruitment of the STAGA complex. We propose that MYC directly binds to the STAGA complex via multivalent contacts of different regions of its TAD with both the TRRAP and GCN5 subunits. GCN5 contacts selectively the MBI-containing 21–108 region and requires the M2 and M3 motifs (this report), while TRRAP may contact both the MBI-containing region and MBII ([19,36], and see Introduction). Although we cannot exclude the possibility that additional STAGA–MYC interactions occur outside the TAD (1–263) domain, both the MBI-containing 1–110 region and MBII are absolutely essential for the MYC–STAGA interaction in vivo [19,36]. Both of these TAD regions are also required for MYC transformation of primary rodent fibroblasts [19], and for efficient induction of cell proliferation and apoptosis by MYC [56]. Notably, however, the MBI-containing 1–100 region, which is absent in MYC-S (short form), is dispensable for MYC transformation of immortalized cells, while MBII is essential [56]; in addition, MBII-independent activities of MYC have also been described [11]. Thus, the functions of the MBI-containing 1–108 region and MBII are not always linked.

While the specific protein–protein interfaces remain to be identified, we have shown that the sequence motifs M2 (residues 54–60 at the core of MBI) and M3 (residues 100–106) are important for the interaction of the TAD (2–108) sub-domain with GCN5 and the STAGA complex in vitro and for optimal interaction of MYC with STAGA in live cells. These motifs could either represent direct contact points for GCN5, since similar M2/3 sequence motifs are found in both p53 and E1a proteins within sequences that interact with GCN5 (or PCAF), and/or they may play a structural role by influencing the conformational dynamics of the 2–108 region. The latter possibility is suggested by NMR studies of the MYC TAD 1–88 segment, indicating that this region is intrinsically disordered but harbors short transiently structured sequences (corresponding to the M1 and M2 motifs), which are involved in dynamic/transient intra-molecular interactions before binding to cofactors [57]. Thus, mutations in M2/3 could potentially also affect the binding of MYC TAD to other cofactors besides GCN5/STAGA, and our studies do not exclude this possibility. We note, however, that the role of the M2/3 motifs is to some extent selective for GCN5/STAGA since one specific amino acid substitution in each motif that impaired MYC TAD binding to GCN5/STAGA had no significant effect on the interaction of other MYC cofactors such as CDK9/P-TEFb and the TIP48 subunit of TIP60 and p400 complexes. Moreover, our results strongly suggest that the physical contacts of GCN5 with the TAD 21–108 sub-region are functionally important in vivo since (i) specific amino acid substitutions within the M2/3 motifs impair acetylation of MYC by GCN5 in cultured cells (Fig. 6B); (ii) GCN5 and the integrity of the M2/3 motifs are important for MYC activation of *TERT* gene transcription in human cell lines (Figs. 7 and 8); and (iii) the M2 and M3 motifs are contained within, respectively, the MBI homology box and the less conserved TAD sequences 93–105 and 94–109, which were previously shown to be important for MYC transformation of primary rat embryo fibroblasts [58–60].

Interestingly, the tumor suppressor protein p53 was shown to interact with STAGA via multivalent contacts involving the GCN5, ADA2B, and TAF9 subunits, but not TRRAP [42]. Taken together with these previous observations, our results suggest that, at least in human cells, different transcription regulators interact with STAGA coregulator complexes in distinct ways — i.e., via different combinations of multivalent contacts with selected protein subunits in the complex. Such diversity of specific activator–coactivator interactions may constitute an additional layer of regulation during the combinatorial control of gene-specific transcription in higher eukaryotes, and could potentially be deregulated in disease states such as cancer. Finally, the finding that the N-terminal TAD of MYC controls acetylation of more C-terminal lysine residues by both GCN5 (Fig. 6B) and p300 [45], and influences MYC binding to DNA/chromatin (Fig. 8B) but not its interaction with MAX, suggests a potential intra-molecular crosstalk between distant MYC

domains and a regulatory role of TAD interactions with HATs and other cofactors beyond a simple recruitment function. Indeed, the SIRT1 deacetylase also interacts with the MBI TAD region [61] and could potentially sterically interfere with GCN5-mediated MYC acetylation, in addition to its reported ability to deacetylate MYC [62,63].

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagr.2014.03.017>.

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