

# An ATPase/Helicase Complex Is an Essential Cofactor for Oncogenic Transformation by c-Myc

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## Summary

The c-Myc transactivation domain was used to affinity purify tightly associated nuclear proteins. Two of these proteins were identified as TIP49 and a novel related protein called TIP48, both of which are highly conserved in evolution and contain ATPase/helicase motifs. TIP49 and TIP48 are complexed with c-Myc *in vivo*, and binding is dependent on a c-Myc domain essential for oncogenic activity. A missense mutation in the TIP49 ATPase motif acts as a dominant inhibitor of c-Myc oncogenic activity but does not inhibit normal cell growth, indicating that functional TIP49 protein is an essential mediator of c-Myc oncogenic transformation. The TIP49 and TIP48 ATPase/helicase proteins represent a novel class of cofactors recruited by transcriptional activation domains that function in diverse pathways.

## Introduction

Oncogenic lesions frequently disrupt the expression or structure of sequence-specific transcription factors, and hence, the molecular basis of oncogenic transformation is intimately tied to the mechanisms of transcriptional regulation. Among the transcription factors with oncogenic potential, c-Myc is one of the most frequent sites of mutation in human cancer (Cole, 1986; Henriksen and Luscher, 1996). Disruption of the c-Myc DNA-binding domain abolishes oncogenic activity, indicating that transformation involves the ability to regulate gene expression (Stone et al., 1987; Amati et al., 1993). Therefore, the activity of c-Myc as an oncoprotein appears to involve the activation of cellular target genes, although the critical target genes through which Myc induces oncogenic transformation, cell proliferation, and apoptosis remain poorly defined (Cole and McMahon, 1999; Dang, 1999).

In lieu of a comprehensive set of target genes, it is of considerable value to understand the general mechanism by which c-Myc regulates gene expression. The amino terminus of c-Myc contains two regions, called Myc homology box I (Mbl) and Myc homology box II (MbII), that are highly conserved between the different Myc family proteins and conserved throughout vertebrate evolution. The MbII domain, spanning amino acids 129–145 in human c-Myc, is necessary for virtually all

c-Myc biological activities, including oncogenic transformation, apoptosis, the ability to block differentiation, and autosuppression of the c-myc promoter (Stone et al., 1987; Freytag et al., 1990; Penn et al., 1990; Evan et al., 1992; Li et al., 1994). However, deletion of MbII has little or no effect on the transactivation of reporter constructs in most transient assays (Kato et al., 1990; Bello-Fernandez et al., 1993; Brough et al., 1995). This suggests that the c-Myc N terminus may have a function that is distinct from the recruitment of basal transcription factors. The evolutionary conservation and essential function of the N terminus suggest that it interacts with critical cofactors that mediate c-Myc function, and initial evidence for this was provided by experiments showing that ectopic expression of the N terminus alone was a dominant inhibitor of oncogenic transformation (Brough et al., 1995).

A critical insight into the mechanism of c-Myc-mediated gene regulation came from the purification of stable complexes between nuclear cofactors and the N terminus. These studies led to the identification of TRRAP, a highly conserved 434 kDa protein with homology to the ATM/PI3-kinase family. TRRAP is essential for Myc-mediated oncogenic transformation, and an ortholog of TRRAP (*TRA1*) is essential for viability in *S. cerevisiae*. Both mammalian TRRAP and *S. cerevisiae* TRA1p are components of the SAGA chromatin-remodeling complex, which contains the histone acetyltransferase GCN5p (hGCN5 or PCAF in mammals) (Grant et al., 1998; McMahon et al., 1998; Ogryzko et al., 1998). We have recently shown that TRRAP mediates the recruitment of hGCN5 by c-Myc and that *in vivo* c-Myc complexes contain histone acetyltransferase activity (McMahon et al., 2000), providing a mechanistic basis for Myc-dependent gene regulation. Recruitment of the SAGA complex is expected to activate target genes through histone acetylation and nucleosome displacement (Kuo et al., 1998; Wang et al., 1998). While the recruitment of the SAGA chromatin-remodeling complex represents an attractive model for Myc-mediated gene regulation, the simple targeting of histone acetylase to chromosomal sites may account for only part of the Myc oncogenic activity. A fusion of the HAT catalytic domain from GCN5 restores only a limited oncogenic activity to a transformation-defective c-Myc mutant (McMahon et al., 2000). This failure could be due to trivial defects in an artificial fusion protein or to activities provided by components of the SAGA complex other than the histone acetylase. However, another explanation for this finding is that the Myc N terminus mediates the recruitment of nuclear cofactors in addition to TRRAP/SAGA and that these other factors are also critical for oncogenic transformation.

Previous work from this lab utilized an affinity purification strategy to isolate the novel c-Myc cofactor TRRAP from nuclear extracts (McMahon et al., 1998). Using a modified protocol, further investigation has led to the identification of four additional nuclear proteins that are tightly associated with the c-Myc N-terminal transactivation domain. Here we report the characterization of

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two of these proteins, called TIP49 and TIP48. TIP49 is a previously described (Kanemaki et al., 1997; Holzmann et al., 1998; Qiu et al., 1998) and highly conserved protein of unknown function that we show acts as an essential cofactor for Myc-mediated oncogenic transformation. In addition, we show that the activity of TIP49 requires its intrinsic ATPase domain, a motif shared between TIP49, TIP48, and well-characterized factors involved in transcription and chromatin remodeling.

## Results

### Affinity Purification of c-Myc Interacting Nuclear Proteins

Expression of the c-Myc N terminus in mammalian cells leads to the formation of a stable nuclear complex that is dependent on the essential MblI domain of c-Myc. Affinity purification demonstrated that one component of this complex is a very large ATM-related cofactor named TRRAP, which was shown to be essential for c-Myc oncogenic activity (McMahon et al., 1998). Our previous purification strategy employed a mild heat treatment (55°C) of the HeLa cell nuclear extract, which preserved the integrity of the TRRAP protein but promoted the more rapid migration of the complex in native gels (T. Hofmann, personal communication). Since the heat treatment was likely to have dissociated important protein cofactors that were bound to c-Myc and/or TRRAP, we repeated the affinity purification with unfractionated nuclear extracts. The affinity purification utilized a protein containing c-Myc amino acids 1–262 fused to a FLAG epitope-tagged GAL4 DNA-binding domain. Proteins associated with the c-Myc N terminus were then eluted using the FLAG peptide in large-scale immunoprecipitation experiments (Figure 1). In addition to TRRAP, the FLAG-GAL4/Myc fusion protein precipitated four other polypeptides with apparent molecular weights of 50 kDa, 48 kDa, 45 kDa, and 41 kDa. Two other proteins that eluted from the beads migrated as a closely spaced doublet with an apparent molecular weight of approximately 60 kDa. The specificity of the latter proteins to the FLAG-GAL4/Myc sample and Western blots using anti-FLAG and anti-Myc antibodies identify the 60 kDa doublet as the FLAG-GAL4/Myc protein used as the affinity substrate in the experiment.

The binding specificity of the affinity-purified protein was assessed in two ways. First, no bands of similar size were present with FLAG-GAL4 protein as an affinity substrate (Figure 1, lane 2). Second, a FLAG-GAL4/N-Myc protein used as an affinity substrate captured the same four proteins from the HeLa nuclear extract, based initially on the pattern of bands in the silver-stained gel (Figure 1, lane 3). The proteins eluted from the affinity beads with FLAG peptide include the FLAG-GAL4/N-Myc protein itself, which migrates in the middle of the HeLa-derived proteins. The identity of the latter was confirmed by Western blot (data not shown). Since both c-Myc and N-Myc bind to the same HeLa nuclear proteins, we infer that these proteins recognize common motifs or structures that are shared by Myc family proteins.

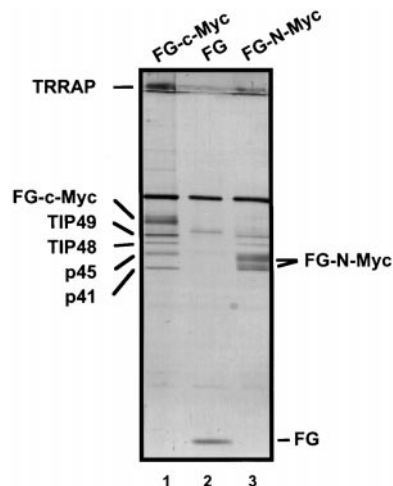


Figure 1. Affinity Purification of Nuclear Proteins Interacting with the Myc Amino Terminus

The FLAG-GAL4/Myc and FLAG-GAL4 proteins were produced as nuclear extracts from SF9 cells and mixed with HeLa nuclear extract and anti-FLAG monoclonal antibody. After precipitation with protein A/G beads, beads were washed and the bound proteins eluted by the addition of FLAG peptide. An aliquot of the eluted material was run on a 10% SDS/polyacrylamide gel, and proteins were visualized by silver staining. Lane 1, elution from the FLAG-GAL4/c-Myc reaction. Lane 2, elution from the FLAG-GAL4 reaction. Lane 3, elution from the FLAG-GAL4/N-Myc reaction. Molecular weight (indicated in kilodaltons) was determined by coelectrophoresis of protein MW marker (Amersham).

### Identification of Two Novel c-Myc N-Terminal Interacting Proteins

The immunoprecipitation experiment was scaled up to obtain approximately 1  $\mu$ g of the 50 kDa and 48 kDa polypeptides, and several peptide sequences were determined by automated microsequencing. Two independent peptides unambiguously identified the 50 kDa polypeptide as the human homolog of TIP49, for which a full-length cDNA was obtained and sequenced (Figure 2). The rat *TIP49* cDNA was originally cloned in a study of proteins interacting with TBP (Kanemaki et al., 1997). Although TIP49 binds to TBP on an affinity column, no in vivo association of these proteins could be demonstrated. The acronym TIP can still apply as transactivation domain interacting protein. Interestingly, TIP49 contains the Walker A and Walker B motifs, which are found in proteins that bind and hydrolyze ATP (Gorbalenya et al., 1989; Schmid and Linder, 1992). TIP49 also has limited homology to the RuvB ATP-dependent DNA helicase, although the Walker A and Walker B motifs are separated by approximately 200 amino acids in TIP49 compared to the closely spaced motifs in RuvB. As an added confirmation that the 50 kDa protein was TIP49, we performed a Western blot using anti-TIP49 antibodies (a generous gift of Dr. Tamura), which detected the same 50 kDa band in the protein isolated through c-Myc affinity (data not shown).

Four independent peptides identified the 48 kDa polypeptide as a novel protein whose closest relative is the TIP49 protein discussed above (Figure 2). We have designated this protein as TIP48 based on this homology, the apparent molecular weight on SDS/PAGE, and the

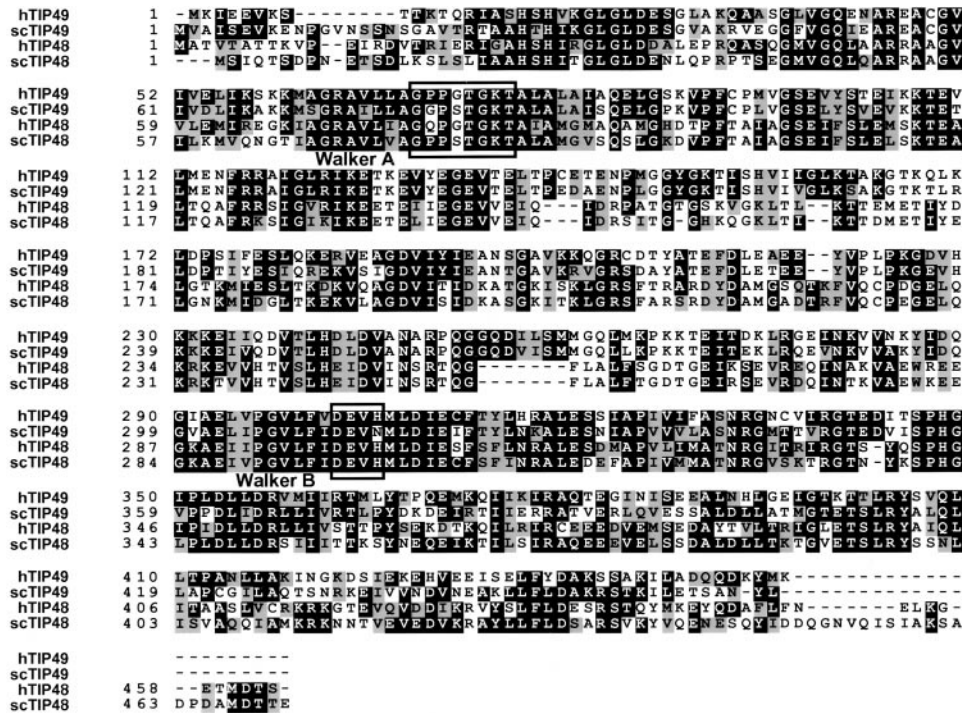


Figure 2. Sequence Comparison between TIP49 and TIP48 Proteins  
Sequences of human TIP49 and TIP48 and *S. cerevisiae* TIP49 (YDR190c) and TIP48 (YPL235w). The Walker A and Walker B motifs are boxed.

characterization presented in this study. We isolated and sequenced the complete human *TIP48* cDNA, and the predicted protein sequence contained all four peptides obtained from microsequencing. The sequence is identical to the cDNA recently described by Kanemaki et al. (1999) and designated *TIP49b*. The *TIP48* cDNA encodes a protein of 463 amino acids while the *TIP49* cDNA encodes a protein of 456 amino acids, indicating that the mobility in SDS/PAGE does not reflect the relative molecular weights. Ectopic expression of TIP48 and TIP49 confirms this relative mobility on SDS/PAGE (data not shown). The amino acid sequences of TIP48 and TIP49 are 45% identical and 60% similar (Figure 2), with the greatest identity within the putative ATPase motifs. Despite their similarity, evidence presented below suggests that these proteins have distinct cellular functions.

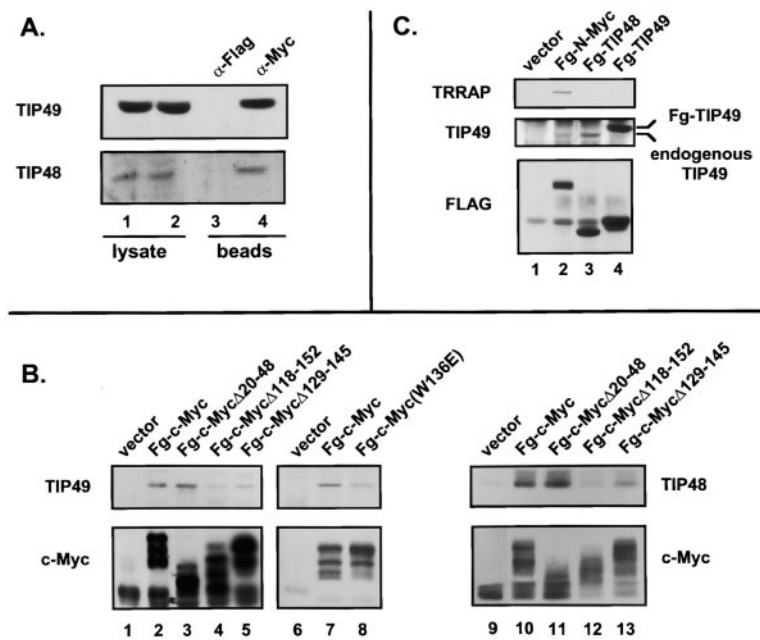
#### c-Myc Interacts with Both TIP48 and TIP49 In Vivo

The affinity purification indicated that TIP49 forms a stable complex with the c-Myc N terminus. To determine whether this association also existed in vivo, we precipitated c-Myc from lysates prepared from 293 cells and probed the precipitates for TIP48 and TIP49 by Western blot (Figure 3A). Immunoprecipitation of c-Myc results in the specific coprecipitation of both proteins. Thus, both TIP48 and TIP49 interact with c-Myc in vivo when these proteins are present at endogenous physiological levels.

Deletion mutants of c-Myc were used to map the regions of the N terminus that are required for interaction with TIP49. Expression vectors for FLAG epitope-tagged wild-type or mutant c-Myc proteins were transiently cotransfected into 293 cells with an expression

vector for HA-tagged TIP49 or HA-tagged TIP48, then the cells were lysed under native conditions, and the Myc proteins were immunoprecipitated with anti-FLAG antibodies. The protein complexes were then resolved on SDS/PAGE and Western blotted using anti-HA antisera to determine the extent of coprecipitation. The same membrane was subsequently probed a second time with anti-Flag antibodies to show equivalent protein expression between the different constructs. The full-length c-Myc protein coprecipitated with HA-TIP49 and HA-TIP48 as expected (Figure 3B, lanes 2, 7, and 10), as did a transformation-defective deletion mutant in the N terminus (*Myc* $\Delta$ 20-48; lanes 3 and 11) that was previously shown to bind poorly to another c-Myc cofactor TRRAP (McMahon et al., 1998). More interestingly, the M<sub>III</sub> region of c-Myc ( $\Delta$ 118-152 and  $\Delta$ 129-145) is necessary for efficient binding to HA-TIP49 and HA-TIP48 (Figure 3B, lanes 4, 5, 12, and 13). Binding was not completely eliminated by a small M<sub>III</sub> deletion, but was substantially reduced with a larger deletion and by a single missense mutation (W136E; lane 8) in M<sub>III</sub> that severely impairs c-Myc oncogenic activity (Brough et al., 1995). Since M<sub>III</sub> is essential for Myc-mediated transformation, this suggested that TIP49 and TIP48 could be critical cofactors as previously shown for TRRAP (McMahon et al., 1998). On the other hand, the binding of TIP49 and TIP48 to Myc $\Delta$ 20-48 distinguishes their binding specificity from that of TRRAP, which is dependent on two c-Myc domains (McMahon et al., 1998).

The M<sub>III</sub>-dependent binding of TIP49 and TIP48 to c-Myc could arise in two ways. TIP49 might bind directly to Myc or it might bind indirectly through TRRAP or



Lanes 4 and 12, IP from cells expressing FLAG-c-Myc( $\Delta$ 118–152). Lanes 5 and 13, IP from cells expressing FLAG-c-Myc( $\Delta$ 129–145). Lane 8, IP from cells expressing FLAG-c-Myc(W136E).

(C) 293 cells were transiently transfected with expression vectors for FLAG-tagged N-Myc, TIP48, or TIP49, as indicated. Lysates were prepared and subjected to immunoprecipitation using anti-FLAG antibody. Precipitated proteins were resolved by SDS/PAGE and Western blotted for either TRRAP (top), TIP49 (middle), or FLAG (bottom). Lane 1, IP from cells transfected with a vector expressing the FLAG epitope alone. Lane 2, IP from cells expressing FLAG-N-Myc. Lane 3, IP from cells expressing FLAG-TIP48. Lane 4, IP from cells expressing FLAG-TIP49.

some other intermediate. To address this question, we assayed for an interaction between TIP49 and TRRAP that is independent of Myc. FLAG-tagged derivatives of N-Myc, TIP48, or TIP49 were expressed transiently in 293 cells and immunoprecipitated with anti-FLAG antibodies, and the immunoprecipitates were analyzed by Western blot for the binding of endogenous TRRAP. FLAG-tagged N-Myc coprecipitated with endogenous TIP49 and TRRAP, whereas no TRRAP coprecipitated with either FLAG-TIP48 or FLAG-TIP49 under the same conditions (Figure 3C). On the other hand, both FLAG-TIP48 and FLAG-TIP49 coprecipitated with endogenous TIP49 (Figure 3C, lanes 3 and 4), discussed in more detail below. We conclude that TIP49 and TIP48 are unlikely to preexist in a complex with TRRAP, but instead that the proteins bind either independently or simultaneously to the c-Myc N terminus.

#### TIP49 Has Distinct Interaction Domains

The transient transfection assays described above provided evidence that TIP48 and TIP49 can form homotypic and heterotypic interactions. To explore this further, different FLAG-tagged and nontagged proteins were transiently expressed, and the binding to endogenous cofactors was assayed following immunoprecipitation with anti-FLAG antibodies. The endogenous TIP48 protein bound to FLAG-tagged TIP49, but not to a nontagged TIP49 used as a control (Figure 4A, compare lanes 2 and 3). In contrast, the endogenous TIP48 protein did not bind to transiently expressed FLAG-tagged TIP48, even though the endogenous and tagged proteins could be resolved on the gel (Figure 4A, lanes 8 and 10). Both FLAG-tagged TIP48 and FLAG-tagged

Figure 3. The In Vivo Interaction between TIP49, TIP48, and c-Myc Requires MbII and Is Independent of TRRAP

(A) 293 cells were lysed and subjected to immunoprecipitation using either anti-FLAG/agarose-conjugated beads or anti-Myc (C33)/agarose-conjugated beads. Precipitated proteins were resolved by SDS/PAGE and Western blotted for TIP49 and TIP48, as indicated. Lanes 1 and 2, lysates alone. Lanes 3 and 4, lysates subject to immunoprecipitation using antibodies indicated.

(B) 293 cells were transiently cotransfected with CMV-driven expression vectors for FLAG-tagged full-length c-Myc or N-terminal mutants of this construct and HA-tagged full-length TIP49 or TIP48. Lysates were prepared and c-Myc proteins immunoprecipitated (IP) with anti-FLAG antibody. Precipitated proteins were resolved by SDS/PAGE and Western blotted for either HA (for TIP49 and TIP48) or FLAG (for c-Myc and c-Myc mutants). Lanes 1, 6, and 9, IP from cells transfected with a vector expressing the FLAG epitope alone. Lanes 2, 7, and 10, IP from cells expressing FLAG-c-Myc. Lanes 3 and 11, IP from cells expressing FLAG-c-Myc( $\Delta$ 20–48).

TIP49 bound to endogenous TIP49 (Figure 4B, lanes 3 and 8). Thus, TIP49 appears to form both homotypic and heterotypic interaction, whereas TIP48 does not appear to form homotypic complexes under the conditions of these experiments.

Deletion mutants of TIP49 provided a preliminary determination of the domains required for complex formation. Deletions were introduced into FLAG-tagged TIP49, and the mutants were transiently expressed in 293 cells. Anti-FLAG immunoprecipitates were then tested for coprecipitation with cotransfected c-Myc, endogenous TIP49, or endogenous TIP48. For c-Myc, the primary domain of interaction maps to the region ( $\Delta$ 136–187) between the Walker A and Walker B motifs (Figure 5B). The interbox deletion does not completely eliminate binding, suggesting that the interaction may extend over a larger region than the one assayed. However, an adjacent deletion ( $\Delta$ 188–259) had no effect on c-Myc binding. Deletion of the Walker A or Walker B motifs had no effect on binding to c-Myc when compared to other mutants and to full-length TIP49.

The same deletion mutants were used to map the TIP49 domains required for homotypic and TIP48 interactions. Since the deletion mutants migrated more rapidly than the wild-type protein, they also provided a much clearer separation of the transiently expressed and endogenous proteins. Deletion of either the Walker A ( $\Delta$ 63–135) or Walker B ( $\Delta$ 290–366) motifs eliminated the binding of the transiently expressed proteins to both endogenous TIP49 (Figure 5C, lanes 3 and 6) and endogenous TIP48 (Figure 5D, lanes 3 and 6). In contrast, neither of the two different deletions within the unique domain that separates the Walker A and Walker B boxes

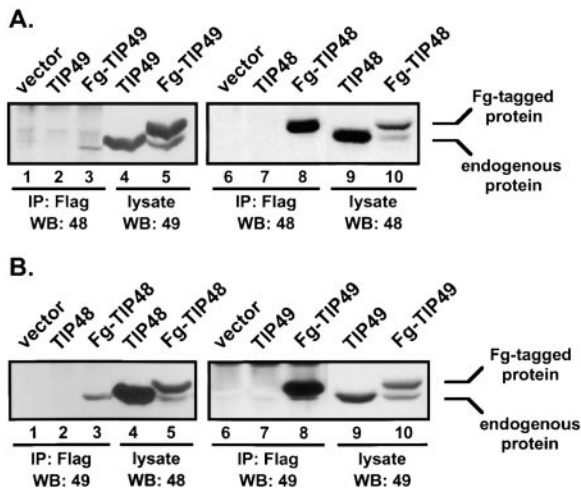


Figure 4. TIP49 and TIP48 Form Homotypic and Heterotypic Interactions In Vivo

(A) 293 cells were transiently transfected with expression vectors producing TIP49, Fg-TIP49, TIP48, and Fg-TIP48 as indicated. Lanes 1, 2, and 3, lysates immunoprecipitated using anti-FLAG antibody and Western blotted for TIP48. Lanes 4 and 5, lysates alone blotted for TIP49 to show expression of constructs used in lanes 2 and 3. Lanes 6, 7, and 8, lysates immunoprecipitated using anti-FLAG antibody and blotted for TIP48. Lanes 9 and 10, lysates alone probed for TIP48.

(B) Same experimental procedure as for (A). Lanes 1, 2, and 3, lysates immunoprecipitated using anti-FLAG antibody and Western blotted for TIP49. Lanes 4 and 5, lysates alone blotted for TIP48. Lanes 6, 7, and 8, lysates immunoprecipitated using anti-FLAG antibody and blotted for TIP49. Lanes 9 and 10, lysates alone blotted for TIP49.

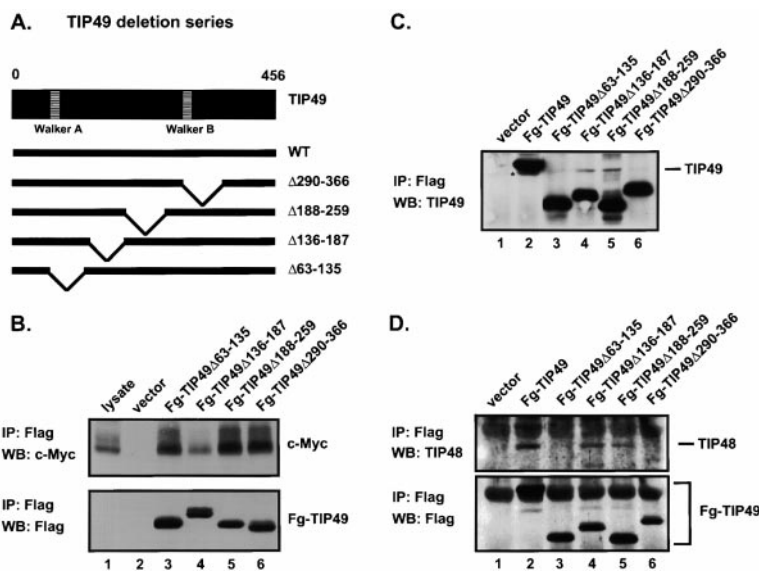
( $\Delta$ 136–187 and  $\Delta$ 188–259) had a major effect on binding to both endogenous TIP49 and TIP48 (Figure 5C and 5D, lanes 4 and 5). These data support the idea that the

ATPase motifs are critical for proper complex formation, although a more subtle mutation of the ATPase motif has no effect on protein–protein interactions (see below). Furthermore, the interaction domain for c-Myc appears to be clearly separable from the domains involved in cofactor–cofactor interactions.

#### A Transdominant Mutant of TIP49 Inhibits Transformation by Myc

Since TIP49 requires the essential and conserved MbII domain to interact with Myc, it suggested that TIP49 might have a role in Myc-mediated transformation. The structural relationship between TIP49 and ATPase/helicase proteins provided a means to test for a functional role in oncogenesis. TIP49 contains limited homology to the RuvB helicase outside the ATPase motifs. Studies of RuvB have previously shown that a single missense mutation at a conserved position in the Walker B box (DEVH→NEVH) created a dominant-negative allele (Mezard et al., 1997). The mutant RuvB protein can still form homomeric complexes and interact with the accessory factor RuvA, but the RuvB/D113N mutant cannot bind and hydrolyze ATP or bind to DNA (Mezard et al., 1997). We introduced the same mutation into the TIP49 Walker B box (D302→N; Figure 6A) and tested its ability to interfere with c-Myc function in a transformation assay. Similar to the RuvB mutant, the TIP49D302N mutant interacts with c-Myc, itself, and TIP48 (Figure 6B and data not shown) to the same extent as TIP49wt, and expression was equivalent to that of the wild-type protein.

The TIP49wt and TIP49D302N expression vectors were cotransfected with expression vectors for c-myc and the oncogene H-rasG12V. Cotransfection of c-myc and H-rasG12V into early-passage rat embryo fibroblasts leads to the formation of oncogenically transformed foci that are strictly dependent on a functional



Fg-TIP49 $\Delta$ 290–366 (lane 6). Lysates were prepared and subjected to immunoprecipitation using anti-FLAG antibody. Precipitated proteins were resolved by SDS/PAGE and Western blotted for TIP49. The asterisk in lane 2 highlights the endogenous TIP49 band running below FLAG-TIP49.

(D) Same experimental procedure as for (C) except that the top panel was probed for TIP48, and the bottom panel was probed for FLAG.

Figure 5. Interactions between TIP49 and TIP48 Map to Different Regions Than Interaction with c-Myc

(A) TIP49 deletion series created by site-directed mutagenesis.

(B) 293 cells were transiently cotransfected with expression vectors producing c-Myc (lanes 1–6), FLAG epitope alone (lane 2), Fg-TIP49 $\Delta$ 63–135 (lane 3), Fg-TIP49 $\Delta$ 136–187 (lane 4), Fg-TIP49 $\Delta$ 188–259 (lane 5), and Fg-TIP49 $\Delta$ 290–366 (lane 6). Lysates were prepared and subjected to immunoprecipitation (except lane 1) using anti-FLAG antibody. Precipitated proteins were resolved by SDS/PAGE and Western blotted for c-Myc (top panel) and FLAG to show equivalent protein expression of the Fg-TIP49 deletion constructs (bottom panel). See Figure 6 for wild-type TIP49 interaction with c-Myc.

(C) 293 cells were transiently transfected with expression vectors producing FLAG epitope alone (lane 1), Fg-TIP49 (lane 2), Fg-TIP49 $\Delta$ 63–135 (lane 3), Fg-TIP49 $\Delta$ 136–187 (lane 4), Fg-TIP49 $\Delta$ 188–259 (lane 5), and

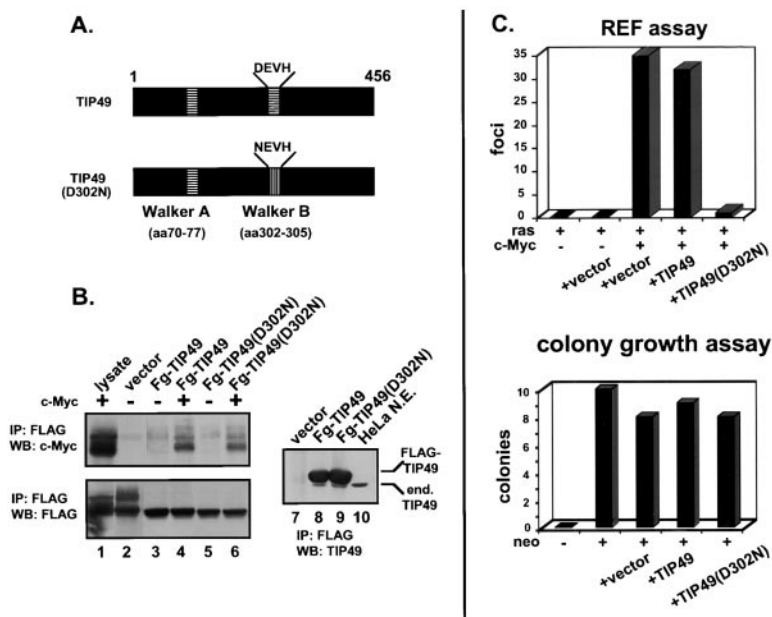


Figure 6. Expression of a Transdominant TIP49D302N Mutant Blocks Transformation of Rat Embryo Fibroblasts by *c-myc* and *H-ras*

(A) Schematic diagram of TIP49. Walker A and Walker B motifs are indicated with the mutation in Walker B used to create the TIP49D302N mutant.

(B) 293 cells were transiently cotransfected with expression vectors producing *c-Myc* (lanes 1, 4, and 6), FLAG epitope alone (lane 2), FLAG-TIP49 (lanes 3 and 4), and FLAG-TIP49D302N (lanes 5 and 6). In a separate experiment (lanes 7–10), 293 cells were transiently transfected with expression vectors producing FLAG epitope alone (lane 7), FLAG-TIP49 (lane 8), and FLAG-TIP49D302N (lane 9). All lysates were prepared and subject to immunoprecipitation using anti-FLAG antibody. Precipitated proteins were resolved by SDS-PAGE and Western blotted for *c-Myc* (top panel, lanes 1–6), FLAG (bottom panel, lanes 1–6), and TIP49 (lanes 7–10).

(C) Primary rat embryo fibroblasts were transfected with expression vectors for *c-Myc*, *H-RasG12V*, TIP49, and TIP49D302N in the

different combinations indicated. The number of transformed foci is shown on the y axis. Duplicate plates of REFs were cotransfected with 1  $\mu$ g of pRSV-*neo* and 3  $\mu$ g of CMV promoter-driven TIP49 or TIP49D302N vectors as indicated. Transfected cells were selected in 400  $\mu$ g/ml G418 for 14 days, at which time the number of colonies per plate was determined. Two plates were assayed for each bar in the graph (bottom). The same colony assay was performed with Rat1 and HeLa cells with similar results (data not shown).

Myc protein. Cotransfection of a TIP49wt expression vector with *c-myc* and *H-rasG12V* had no effect on focus formation. In contrast, cotransfection with the TIP49D302N mutant led to a complete inhibition of Myc-mediated transformation (Figure 6C). It was important to determine whether the inhibitory effect of the TIP49D302N mutant was specific to oncogenic transformation or the result of a general growth inhibition or toxicity. Both the TIP49wt and TIP49D302N proteins were assayed for toxicity or nonspecific growth inhibition by determining the efficiency of G418-resistant colony formation in several different cell lines. When cotransfected with pRSV-*neo*, equal numbers of G418-resistant colonies of early-passage rat embryo fibroblasts were obtained with all expression vectors when compared to the empty vector control (Figure 6C), indicating that there was no cellular toxicity or growth inhibition from the dominant-negative mutant. There was also no inhibition of colony growth with immortalized Rat1 fibroblasts or HeLa cells (data not shown). The TIP49D302N mutant also has no effect on the cell cycle distribution of transiently transfected Rat1 fibroblasts using flow cytometry (data not shown). We infer from these results that a basal level of wild-type TIP49 protein is a rate-limiting cofactor in Myc-mediated transformation, and the predicted ATPase-deficient mutant interferes with the endogenous pool of functional protein. On the other hand, the lack of general growth inhibition by the mutant implies that endogenous TIP49 function is not rate limiting for normal cell growth. However, ectopic expression of the TIP49D302N mutant protein is unlikely to induce a complete loss of function, and a complete loss of function might be incompatible with cell growth (see below).

#### TIP49 and TIP48 Expression Are Suppressed by *c-Myc* Loss of Function

TIP49 has previously been shown to be ubiquitously expressed (Bauer et al., 1998; Holzmann et al., 1998; Kanemaki et al., 1999); however, it was of interest to explore any potential link between *c-Myc* expression and the expression of the cofactors that mediate *c-Myc* function. Serum stimulation of Rat1 fibroblasts was found to induce both *TIP49* and *TIP48* mRNA levels approximately 4- to 5-fold within 3 hr after serum addition to quiescent cells (Figure 7A). Interestingly, *TIP49* mRNA induction is dependent on the expression of the endogenous *c-Myc* protein since induction is abolished in a cell line in which the *c-myc* gene has been knocked out by homologous recombination (Mateyak et al., 1997). The Myc-dependent defect in *TIP49* and *TIP48* expression is also evident in log phase cells, in which the *c-myc* null cells have a consistent 3- to 4-fold reduction in both mRNAs (Figure 7A, lane 12). The Myc dependence of TIP49 and TIP48 expression extends to the protein level since the *c-myc* null cells have a corresponding 3- to 4-fold decrease in both proteins. Reconstitution of either *c-Myc* or *N-Myc* into the *c-myc* null cells restores the level of TIP49 and TIP48 proteins to that found in the parental cell line from which the *c-myc* null line was derived (Figure 7B). Thus, the expression of these two Myc cofactors is dependent on Myc itself, although further studies will be required to determine whether this is a direct or indirect regulatory link.

#### The ATPase Motif in *S. cerevisiae* TIP49 Is Essential for Viability

A search of GenBank revealed that the human TIP49 and TIP48 proteins have distinct orthologs in the yeast

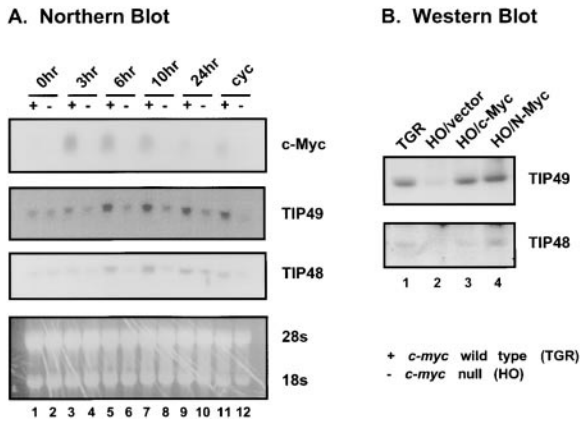


Figure 7. Expression of TIP49 and TIP48 Is Cell Cycle Regulated and Partially Dependent on c-Myc Expression

(A) *c-myc* diploid (+) and *c-myc* null (-) cells were rendered quiescent by culturing in 0.1% serum for 48 hr and were then induced to reenter the cell cycle with 10% serum medium. Total RNA was isolated at the indicated time points as well as from exponentially growing cells (lanes 11 and 12). The same blot was probed multiple times with the probes indicated. Lanes 1, 3, 5, 7, 9, and 11 contain RNA from *c-myc* diploid (+) cells. Lanes 2, 4, 6, 8, 10, and 12 have RNA from *c-myc* null (-) cells. The ethidium bromide-stained RNA gel shows equivalent loading of samples (bottom).

(B) Lysates were prepared from log phase cells: lane 1, *c-myc* diploid (+) cells (TGR); lane 2, *c-myc* null (-) cells reconstituted with empty vector; lane 3, *c-myc* null (-) cells reconstituted with c-Myc; and lane 4, *c-myc* null (-) cells reconstituted with N-Myc. Lysates were run on SDS/PAGE and Western blotted for TIP49 and TIP48, as indicated.

*S. cerevisiae*, which correspond to the largely uncharacterized ORFs YDR190c and YPL235w, respectively. Both the human and *S. cerevisiae* proteins have virtually identical Walker A/B motifs (Figure 2). Although the human TIP49 and TIP48 proteins are closely related, the optimal alignment indicates that the yeast ORF YDR190c is more closely related to TIP49 than to TIP48. hTIP49 is 69% identical and 78% similar to yeast YDR190c, but only 43% identical/55% similar to YPL235w. hTIP48 is 67% identical/77% similar to its *S. cerevisiae* counterpart. Because of this extensive homology, we have designated YDR190c as *scTIP49* and YPL235w as *scTIP48*. The significant evolutionary conservation of these proteins suggests that they mediate important cellular functions, and distinct orthologs exist in all eukaryotes as well as archaeobacteria (Makino et al., 1999). A previous study showed that *scTIP49* and *scTIP48* are essential genes for vegetative growth (Bauer et al., 1998; Qiu et al., 1998; Kanemaki et al., 1999), and independent gene knockouts generated in our study confirm this finding (data not shown).

The existence of an essential and highly conserved yeast ortholog of human TIP49 provided a means to test the function of the TIP49D302N mutant, which was a potent dominant inhibitor of Myc-mediated transformation. The sequence of the Walker B box and DEVH motif are identical between yeast and human TIP49 proteins, so the equivalent D→N mutation was introduced by site-directed mutagenesis into the yeast gene (*scTIP49D311N*) on a plasmid expression vector. The plasmid was transformed into a diploid *tip49Δ* deficiency

Table 1. ATPase motif in *S. cerevisiae* TIP49 Is Essential for Viability

	Tetrads Dissected	Viable Spores			
		1	2	3	4
Wild type	12			1	11
<i>tip49Δ</i>	14	3	11		
<i>tip49Δ</i>	10				10
<TIP49>					
<i>tip49Δ</i>	15	2	13		
<TIP49D311N>					
<i>tip48Δ</i>	14	2	12		
<i>tip48Δ</i>	20	4	16		
<TIP49>					

Wild-type strain: YPH501 MATa/α *ura3-52/ura3-52, lys2-801/lys2-801, ade2-101/ade2-101, trp1-Δ63/trp1-Δ63, his3-Δ200/his3-Δ200, leu2-Δ1/leu2-Δ1*. *tip49Δ*: *tip49Δ::HIS3*. <TIP49>: 2μ plasmid expressing wild-type *scTIP49* (Genestorm clone from Invitrogen). <TIP49D311N>: same as <TIP49> but with D311N mutation. *tip48Δ*: *tip48Δ::HIS3*.

strain (*tip49Δ::HIS3;TIP49*). After sporulation, the tetrads were dissected and colony growth was assessed. Only 2 out of 4 spores for each tetrad formed viable colonies, and no colonies inherited the *HIS3* marker, indicating that the *scTIP49D311N* mutant failed to rescue the *tip49Δ* deficiency (Table 1). This contrasts with the wild-type *scTIP49* vector that rescues with nearly 100% efficiency (Table 1). This finding supports the original rationale that the hTIP49D302N mutation would create a nonfunctional protein. The wild-type *scTIP49* plasmid cannot rescue a *tip48Δ*-deficient strain (Table 1), indicating that the two genes have nonredundant functions.

## Discussion

The biochemical purification of nuclear proteins that bind with high affinity to the Myc oncoprotein has identified several novel cofactors associated with transcriptional activation. The TIP49 and TIP48 proteins are examples of transcriptional cofactors with ATPase and helicase activities. Since the proposed cellular target genes with demonstrated Myc/Max-binding sites are activated by Myc (Cole and McMahon, 1999; Dang, 1999), the functional data presented here indicates that the recruitment of TIP49 and potentially other components of a complex are essential for the activation of critical targets. The dependence on the MblI domain of c-Myc for interaction with TIP49 provides a link with c-Myc biological activity, including oncogenic transformation, induction of apoptosis, and blocking differentiation. Recent data suggest that many proposed c-Myc target genes are not dependent on c-Myc for expression in log phase cells and only minimally dependent on c-Myc (<2-fold) during growth factor stimulation (Bush et al., 1998). The most straightforward interpretation of these inconsistent findings is that some but not all promoters that are activated by c-Myc will require the recruitment of the TIP49 protein.

TIP49 was originally identified as a TBP interacting protein using TBP as an in vitro affinity matrix for rat liver nuclear extracts (Kanemaki et al., 1997). However, there was no observable association between TIP49 and

TBP *in vivo*. We also do not detect any TBP in the affinity-purified proteins that bind to the Myc N terminus (M. A. W., unpublished observation). TIP49 was subsequently cloned in a two-hybrid screen using the replication protein 3 as bait and called RUVBL1 due to the limited homology with RuvB (Qiu et al., 1998). However, as with TBP, no *in vivo* association of TIP49 with RPA3 has been demonstrated, and there are no data implicating TIP49 for a role in DNA replication. Of relevance to the present study was the observation that a portion of the cellular TIP49 could be isolated in chromatographic fractions containing RNA polymerase II (Qiu et al., 1998). A third study found that TIP49 was associated with the nuclear matrix (Holzmann et al., 1998).

The finding that TIP49 can also bind to  $\beta$ -catenin and LEF-1/TCF (Bauer et al., 1998) supports a role for TIP49 as a cofactor that is likely to function with diverse transcription factors. Despite the identification of TIP49 through binding studies with different nuclear components, no functional role for TIP49 in these systems has previously been established. The observation that the TIP49D302N allele inhibits Myc oncogenic activity demonstrates that TIP49 is an essential nuclear cofactor in at least the Myc transcription factor pathway. Since the analogous mutation is nonviable in yeast and it targets the conserved Walker B motif, the data imply that Myc-mediated oncogenesis requires the ATPase activity of TIP49. Even though the predicted ATPase-deficient TIP49 inhibits oncogenic transformation, it is not overtly toxic since there is no inhibition of drug-resistant colony formation in several cell types. It is likely that ectopic expression of the TIP49D302N protein creates only a partial loss of function within a cell and that this partial loss of function is only rate limiting when high levels of Myc activity are demanded. The inviability of the yeast strain with the analogous *TIP49D311N* mutation argues that the exclusive expression of the TIP49D302N protein in mammalian cells would also be incompatible with cell growth. Nevertheless, a direct role for TIP49 in the transcriptional activation of any specific Myc target gene is only inferred at this stage, but not yet readily assayed. Coexpression of TIP49wt or TIP49D302N with Myc neither augments nor inhibits the activation or repression of several reporter constructs (J. Skaar, personal communication). A similar finding has been reported for TIP49 in the  $\beta$ -catenin/LEF-1 system. The activity of TIP49 may only be apparent with currently uncharacterized promoters or with chromosomal target sites that are not adequately recapitulated by DNA transfection.

The immunoprecipitation experiments suggest that TIP49 and TIP48 may interact directly and that this interaction requires the Walker A and Walker B boxes associated with ATPase activity. Although we have not directly shown that this interaction is necessary for the function of either protein, it is interesting to speculate that TIP49 and TIP48 may form a functional multimeric complex that is brought to a promoter by interacting with c-Myc. While this manuscript was in preparation, the rat TIP49 protein was shown to have intrinsic ATPase activity that is stimulated by single-stranded DNA and to have intrinsic 3'→5' helicase activity (Makino et al., 1999). The TIP48 protein was also identified recently through its homology to TIP49 and named TIP49b. TIP48 (TIP49b) was also shown to have ATPase and helicase activity,

but the polarity of the helicase was 5'→3' (Kanemaki et al., 1999). Both ATPases are found in large complexes of 600–2000 kDa in nuclear extracts, but the composition of these complexes is presently unknown (Kanemaki et al., 1999). We did not detect any other proteins bound to the c-Myc N terminus with the same stoichiometry as TIP49 and TIP48, except for TRRAP and two proteins of 45 and 41 kDa apparent molecular mass. The latter will be described in a separate report, but we have no evidence that the TIP49 and TIP48 ATPases function in conjunction with TRRAP, p45, or p41.

The precise function of TIP49 and TIP48 will require further study, but the importance of ATPase/helicase motifs has been established for several cofactors that are involved in transcription. The transcriptional cofactor with the most obvious analogy is TFIIH, a multiprotein complex of which two subunits have intrinsic helicase activity (Sung et al., 1993; Ma et al., 1994; Schaeffer et al., 1994). TFIIH functions both as a general transcription factor and in nucleotide excision repair, and the helicase subunits of TFIIH have opposite helicase polarity (Drapkin et al., 1994; Sung et al., 1996). Intriguingly, the chromosomal sites through which c-Myc has been reported to activate target genes are frequently within exons or introns, regions that are transiently denatured by the transcription complex (Dang, 1999). While a function for TIP49 and TIP48 linked to helicase activity is attractive, other transcriptional cofactors with ATPase/helicase motifs are thought not to promote direct DNA strand displacement but instead to modify DNA-protein complexes. Two relevant examples of such factors are SWI2/SNF2 and Mot1 (Cote et al., 1994; Peterson et al., 1994; Collart, 1996; Madison and Winston, 1997). Despite the homology to helicases, no DNA denaturing activity has been detected for SWI2/SNF2, and it is presumed that the function of this factor is to modify DNA/nucleosome interactions (Schnitzler et al., 1998; Phelan et al., 1999). The nucleosome remodeling activity of the related RSC complex is also dependent on the ATPase activity of the related Sth1p (Laurent et al., 1992; Cairns et al., 1996). Some parallels can be drawn between SWI2/SNF2 and another nuclear factor with ATPase/helicase homology, MOT1p (Auble et al., 1994). The primary activity of Mot1, which is essential for yeast viability, appears to be the disruption of TBP/DNA complexes, again with no detectable DNA denaturation activity (Auble et al., 1994). Given these diverse examples, it is not possible a priori to predict the substrate for a TIP49/TIP48 ATPase-dependent function. If the precise function of TIP49 and TIP48 is preserved in evolution, the existence of a potential TIP49 ortholog in archaeobacteria suggests that the substrate will also be a highly conserved nuclear structure or protein-DNA complex. On the other hand, it is equally possible that TIP49 activity is used in multiple DNA-protein complexes to modify diverse substrates that vary between organisms or cell types. A combined biochemical and genetic approach to TIP49 and TIP48 functions should provide important insight into the essential role for these proteins in cell growth and their utilization as cofactors in Myc-mediated oncogenic transformation. The link to oncogenesis raises the intriguing possibility of developing pharmacologic agents to inhibit Myc function through the inhibition of the enzymatic activity of an essential cofactor.

## Experimental Procedures

**Biochemical Purification of c-Myc-Associated Nuclear Proteins**  
Large-scale affinity chromatography was performed with FLAG-GAL4, FLAG-GAL4/c-Myc, or FLAG-GAL4/N-Myc added to HeLa nuclear extract and supplemented with anti-FLAG antibody as described previously (McMahon et al., 1998). For microsequencing, eluted proteins were resolved by 10% SDS-PAGE, visualized by Coomassie staining, excised, and submitted to Harvard University Microchemistry Facility. Peptide sequences GLGLDESLAK and TALALIAQELGSK identified TIP49, and peptide sequences VLI-AGQPGTGK, TEALTOAFR, EVVHTVSLHEIDVINSR, and TQG(YV)\*ALFSGDTGEIK identified TIP48. The last peptide sequence differs from the cDNA, which encodes amino acids FL instead of YV. Full-length human cDNAs were obtained by ordering ESTs from ATCC (TIP49: EST185194) and Genome Systems (TIP48: ym88h02.r1).

## Transfection and Immunoprecipitation

293 cells were cultured in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% fetal calf serum (GIBCO-BRL). Cells were transfected using the calcium phosphate method and lysed using either E1A lysis buffer (Harlow et al., 1986) or F buffer (Sommer et al., 1998). To determine the level of protein production, lysates were analyzed by Western blotting with anti-FLAG monoclonal antibody. For immunoprecipitations, lysates were incubated with anti-FLAG in conjunction with protein G beads. Precipitates were then analyzed by Western blotting with anti-FLAG, anti-HA, anti-TIP49 (generated against a C terminal peptide: LFYDAKSSAKI-LADQQDKYMK), anti-TIP48 (generated against a C terminal peptide: MKEYQDAFLFNLKGETMDS), and anti-TRAAP (McMahon et al., 1998). For the in vivo interaction experiment, 293 cells were lysed in F buffer and subjected to immunoprecipitation using anti-Myc (C33)-conjugated beads (Santa Cruz Biotechnology) or anti-FLAG-conjugated beads (a gift of Debbie Morrison). Antibody detection was performed using ECL (Amersham).

## Transformation Experiments

Transformation assays involving rat embryo fibroblasts were performed as described previously (Brough et al., 1995). Transfections included a CMV promoter-driven FLAG epitope-tagged *c-myc* expression vector (1  $\mu$ g) and *H-ras*G12V (1  $\mu$ g), supplemented with FLAG-TIP49 (0.5  $\mu$ g), FLAG-TIP49D302N (0.5  $\mu$ g), or empty vector. Transfections were performed in triplicate, and the data from multiple experiments were averaged.

For colony growth assays, early-passage primary rat fibroblasts cells, HeLa cells, or Rat1 fibroblasts were transfected with the same CMV-driven cDNA expression vectors used in the REF transformation assays. Three micrograms of each construct was transfected into the cells along with RSV-*neo* by the calcium phosphate method. Transfected cells were selected in 400  $\mu$ g/ml G418 (GIBCO-BRL) for 14 days, at which time the number of colonies per plate was determined.

## Northern and Western Blot Analysis

TGR-1 (*c-myc* diploid) and HO15.19 (*c-myc* null) cells (Mateyak et al., 1997) were cultured as described (Bush et al., 1998). *c-myc* reconstituted cells were created by infecting HO15.19 cells with the LXSH retrovirus containing a full-length mouse *c-myc* cDNA or full-length mouse *N-myc* cDNA. Lysates were prepared, resolved, and probed as described above.

## Yeast Knockout and Rescue Experiments

Primers were designed having homology to *TIP49* or *TIP48* and *HIS3* to PCR-amplify a knockout DNA fragment. The PCR products were gel purified and transformed into YPH501 MAT $\alpha$ / $\alpha$  (see Table 1 for genotype) to create deletions by gene replacement. Diploids were sporulated and their meiotic progeny were analyzed. Rescue experiments were performed by transforming *tip49* $\Delta$  and *tip48* $\Delta$  strains with a Genestorm yORF expression vector producing the *S. cerevisiae* *TIP49* homolog, *YDR190c* (Invitrogen, cat# YDR190CY). The *TIP49* cDNA in this expression vector was used to create the *scTIP49D311N* mutant by site-specific mutagenesis.

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