

TIP49, but not TRRAP, modulates c-Myc and E2F1 dependent apoptosis

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We previously described two nuclear cofactors, TRRAP and TIP49, that have functional roles in Myc-mediated oncogenesis. We have now expanded the analysis of these Myc-associated cofactors to investigate their roles in apoptosis and cell proliferation. Although TRRAP and TIP49 are both essential for transformation, TIP49 modulates c-Myc-mediated apoptosis whereas disruption of TRRAP activity has no apparent effect on apoptosis. We extended our analysis of TIP49 to show that it also binds to the E2F1 transactivation domain and modulates both transforming and apoptotic activities. These results indicate that individual cofactors differentially potentiate c-Myc and E2F1 functions.

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Introduction

The Myc and E2F transcription factor families are among the most frequently disrupted networks in human and animal cancers (Henriksson and Luscher, 1996; Sladek, 1997). Although the sets of target genes involved are still under investigation, both transcription factor families play direct role in G1→S progression by controlling growth and DNA replication. In addition to their role in cell growth, the Myc and E2F families also have the potential to induce programmed cell death or apoptosis, which may oppose the outgrowth of cancer cells that suffer mutations in either network (Phillips *et al.*, 1997; Prendergast, 1999). The mechanisms through which a transcription factor can switch from promoting cell growth to promoting cell death is of great interest in cancer cell biology.

The analysis of Myc protein function has revealed two domains that are primarily required for all biological activities. The Myc C-terminal DNA binding domain is required for both oncogenic and apoptotic activities, establishing a requirement for the recognition of chromosomal sites (Amati *et al.*, 1993; Stone *et al.*, 1987). Myc biological activities also require the N-terminal transactivation domain which functions to

recruit nuclear cofactors to specific sites to modulate transcription and/or alter chromatin structure (Sakamuro and Prendergast, 1999). A number of studies have identified an evolutionarily conserved sequence called Myc homology Box II (MBII) as essential for oncogenic and apoptotic activities, as well as for blocking differentiation (Evan *et al.*, 1992; Freytag *et al.*, 1990; Penn *et al.*, 1990; Stone *et al.*, 1987). Studies centered on understanding the function of MBII led to the purification of TRRAP, a 430 kDa nuclear protein with homology to the ATM/PI-3 kinase family (McMahon *et al.*, 1998). Inhibition of TRRAP synthesis or function blocks Myc-mediated oncogenic activity, establishing an essential role for TRRAP in c-Myc activity (McMahon *et al.*, 1998; Park *et al.*, 2001). TRRAP provided a key mechanistic link to c-Myc function when it was found that TRRAP was a component of the SAGA chromatin modifying complex in both yeast and mammalian cells (Grant *et al.*, 1998; McMahon *et al.*, 2000; Saleh *et al.*, 1998; Vassilev *et al.*, 1998). TRRAP (Tra1p) is also part of separate chromatin modifying complexes containing Esalp or TIP60 in yeast and humans, respectively (Allard *et al.*, 1999; Ikura *et al.*, 2000). These complexes regulate gene expression through enzymatic subunits with histone acetylation activity, either GCN5/PCAF, Esa1p or TIP60. The acetylation of histones stimulates transcription by reducing the affinity of nucleosomes for DNA and facilitating the access of other transcription factors. It was subsequently shown that c-Myc recruits the hGCN5 histone acetyltransferase and that this enzyme is critical for Myc oncogenic activity (McMahon *et al.*, 2000). More recently, TRRAP has been found in a complex with p400, but this complex lacks HAT activity (Fuchs *et al.*, 2001).

Affinity purification of other nuclear factors that bind tightly to the c-Myc N-terminus identified the TIP49 and TIP48 proteins as critical cofactors (Wood *et al.*, 2000). Like TRRAP, TIP49 and TIP48 are highly conserved in evolution and essential for viability in yeast, but the latter proteins contain ATPase/helicase motifs rather than histone modifying activity. Mutation of the ATPase motif in TIP49 creates a dominant inhibitor of c-Myc oncogenic activity, establishing a critical role for this enzyme in Myc function (Wood *et al.*, 2000). The precise role of these ATPase/helicase family proteins in cell physiology requires further study, but they have also been reported to bind to other transcription factors (Bauer *et al.*, 1998; Cho *et al.*, 2001) and have recently been shown to be in a chromatin remodeling complex in yeast

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(Shen *et al.*, 2000) as well as in a complex with TIP60, a histone acetyltransferase (Ikura *et al.*, 2000) as well as both TRRAP and p400, an E1A-associated factor (Fuchs *et al.*, 2001).

Studies of the adenovirus E1A protein led to the identification of another major transcription factor network with striking parallels to the Myc family. Like Myc, E1A can transform and immortalize cells, stimulate cell proliferation, induce apoptosis, and block differentiation (Nevins, 1995; Zantema and van der Eb, 1995). However, unlike Myc, E1A is not a DNA binding protein but instead acts by modulating the activity of the E2F transcription factors. The E2F proteins share all of the biological activities of E1A and Myc, establishing them as the direct mediators of E1A function. Interestingly, E1A does not bind to the E2F proteins themselves, but instead acts by disrupting or modifying the cofactors that are recruited by E2F to chromosomal sites. E1A sequesters the Rb tumor suppressor, preventing it from masking the E2F transactivation domain and converting E2F from an activator into a repressor (Dyson, 1998). E1A also binds to p300/CBP to suppress the mdm2 negative feedback loop and induce p53 apoptotic function (Thomas and White, 1998). Yet E2F1 can induce apoptosis in cells that are genetically deficient for both Rb and p53, indicating that there are multiple apoptotic pathways (Hsieh *et al.*, 1997; Phillips *et al.*, 1997). An *in vivo* role for E2F1 in mediating apoptosis has also been observed in knockout mouse strains (Fields *et al.*, 1996). The overlap between the E2F and Myc families extends to the fundamental enzymatic mechanisms since Rb repressor activity is linked to histone deacetylase and the E2F transactivation domain binds to TRRAP (McMahon *et al.*, 1998). TRRAP and hGCN5 mediate at least part of the E2F transactivation function (Lang *et al.*, 2001). E2F can also recruit the p300/CBP coactivator, which has inherent HAT activity (Martinez-Balbas *et al.*, 2000). Thus a nexus of key nuclear cofactors has emerged as essential mediators of oncogenic transformation and cell proliferation.

Here we have investigated the differential activity of nuclear cofactors in mediating oncogenic transformation, apoptosis and cell proliferation by both the Myc and E2F transcription factors. We find that individual cofactors may have dominant roles in directing Myc and E2F activity toward either cell growth or cell death.

Results

Dominant inhibition of TIP49, but not TRRAP, promotes Myc-dependent apoptosis

Previous studies have investigated the role of different nuclear cofactors in Myc- and E1A-mediated oncogenic transformation (McMahon *et al.*, 1998; Wood *et al.*, 2000), however both transcription factor families also function in cell proliferation and apoptosis. Since oncogenic transformation by Myc is dependent on both TIP49 and TRRAP complexes, we were interested in

determining if these same cofactor complexes also had a prominent role in other biological activities. Stable constitutive expression of c-Myc induces apoptosis in several different cell types when they are starved for growth and survival factors. We analysed a cell line (Myc3) in which a c-myc cDNA was transduced by retroviral vector into a rat fibroblast cell line that has no endogenous c-Myc expression through gene knockout (Bush *et al.*, 1998; Mateyak *et al.*, 1997). Reconstitution of c-Myc expression restores the cell cycle defect that arises through c-myc knockout, but also induces apoptosis when the cells are starved in 0.1% serum (Figure 1a). On the other hand, the parental c-myc null cells have no detectable apoptosis when serum starved (Chang *et al.*, 2000). We tested the TIP49(D302N) mutant along with TIP49wt for any effects on apoptosis. In the presence of serum factors, Myc3 cells exhibit no spontaneous apoptosis, and transfecting with TIP49 or TIP49D302N did not induce apoptosis (Figure 1a, white bars). On the other hand, starving the cells for 24 h induced a low level of apoptosis, which was greatly enhanced by transfection of TIP49 and TIP49(D302N) (Figure 1a, black bars). Stable expression of a c-Myc(Δ 129–145) mutant in the c-myc null cells failed to induce apoptosis and there was no potentiation by TIP49 or TIP49(D302N) (data not shown). Thus, cells with stable misregulation of c-Myc are sensitized to apoptosis by TIP49.

To further study the activities of different cofactors in apoptosis, we transfected an expression vector for c-Myc along with TIP49 or TRRAP into the parental rat fibroblast cell line with wild type c-myc genes. A membrane-localized green fluorescent protein (GRP) was also included to mark the transfected cells. Cells were harvested and analysed for apoptosis by the appearance of cells with a sub-G1 DNA content. In the presence of serum, c-Myc did not induce apoptosis and the addition of wild type or dominant inhibitory forms of TIP49 and TRRAP had no effect (data not shown). Starving the cells in 0.1% serum after transfection did not lead to consistent apoptosis with wt c-Myc (Figure 1b, black bars), unlike stable cell lines with constitutive c-Myc expression. We attribute this to a difficulty in achieving high enough c-Myc levels at the proper cell density to induce apoptosis in transient assays. Supplementing the transfections with the dominant negative TIP49(D302N) mutant significantly enhanced apoptosis with c-Myc to 30% of the transfected cells, whereas wt TIP49 had a more modest effect and enhanced apoptosis to 9% (Figure 1b, black bars). As expected, the Myc(Δ 129–145) mutant failed to induce apoptosis either alone or when cotransfected with either wtTIP49 or TIP49D302N (Figure 1b, shaded bars). Thus, disruption of TIP49 activity by over-expressing the ATPase domain mutant greatly potentiates the apoptotic activity of the c-Myc transcription factors even in transient assays.

Since the disruption of TRRAP cofactor activity by ectopic expression of certain protein domains inhibits oncogenic transformation (McMahon *et al.*, 1998), we also tested if these same vectors could block or enhance

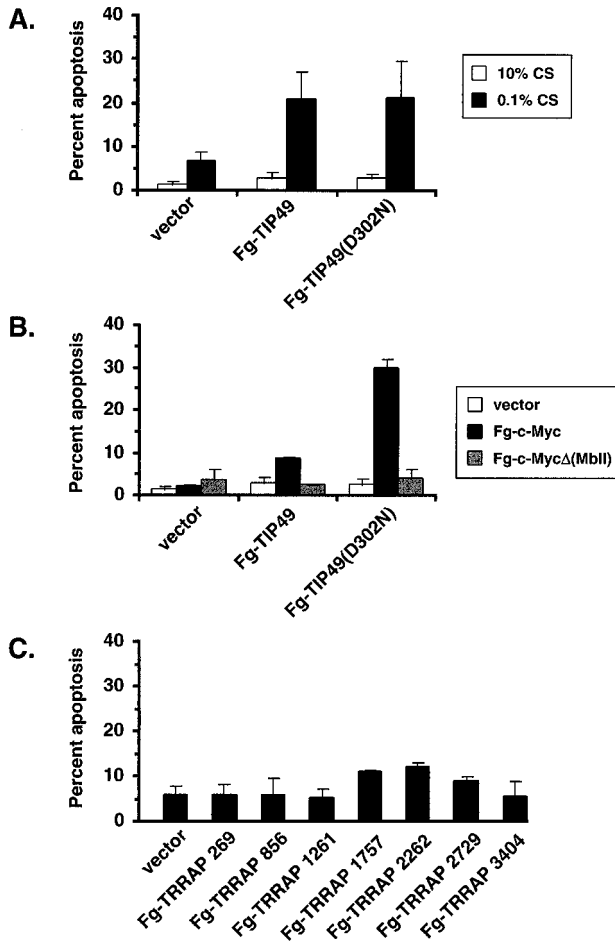


Figure 1 TIP49D302N mutant, but not TRRAP, promotes Myc-dependent apoptosis (a) Myc3 cells were transiently transfected with 5 μ g of vector expressing the FLAG epitope alone, FLAG-TIP49 or FLAG-TIP49D302N plus 0.5 μ g of a construct expressing GFP. The cells were cultured in DMEM with either 10% FCS or 0.1% FCS, as indicated. Both floating and adherent cells were harvested and analysed by flow cytometry to determine the percentage of apoptotic cells. The percentage of GFP-positive cells with a sub-G1 DNA content is plotted on the y-axis. (b) TGR/Rat1 cells were transiently transfected with 5 μ g of vector expressing the FLAG epitope alone, FLAG-TIP49 or FLAG-TIP49(D302N) in conjunction with vector, FLAG-c-Myc or FLAG-c-Myc Δ MbII plus 0.5 μ g of a construct expressing GFP. The cells were starved in DMEM with 0.1% FCS and analysed as in (a). The percentage of apoptosis of the transfected cells was determined by subtracting the background level of apoptosis in GFP-negative cells (2–7%) from the apoptosis achieved in the GFP-positive cells. (c) Myc3 cells were transiently transfected with 5 μ g of the indicated FLAG–TRRAP vectors (described in Materials and methods) plus 0.5 μ g of a GFP expression vector. The cells were starved in DMEM with 0.1% FCS and analysed as in (a)

apoptosis. An extensive series of TRRAP protein segments was cotransfected with c-Myc, including two segments that were previously found to inhibit transformation (Fg-TRRAP(1261–1579) and Fg-TRRAP(3404–3830)). All of the TRRAP segments were expressed well in transient assays ((McMahon *et al.*, 1998) and data not shown). Unlike TIP49, none of the TRRAP segments enhanced or suppressed apopto-

sis by c-Myc (Figure 1c). Thus, the TIP49 and TRRAP nuclear cofactor complexes appear to have distinct roles in apoptosis and oncogenesis.

TIP49 binds to the E2F1 transactivation domain

The E2F transfection factor family has activities that parallel those of c-Myc, and E2F1 is the major downstream effector of E1A oncogenic activity. Since E2F1 binds to the TRRAP cofactor complex through the C-terminal transactivation domain (McMahon *et al.*, 1998), we were interested to determine if E2F1 also binds to the TIP49 complex and if TIP49 was required for biological activity. FLAG-epitope tagged expression vectors for E2F1 and a deletion mutant lacking the transactivation domain were transiently transfected into 293 cells and the binding to endogenous TIP49 was assayed by Western blot. Co-precipitation of the FLAG-tagged E2F1 protein with endogenous TIP49 was readily detected compared to the control vector, whereas the E2F1 deletion mutant that lacks the transactivation domain was substantially reduced in binding (Figure 2a, lanes 2–5). Binding to the deletion mutant was not entirely eliminated, indicating that other domains of E2F1 or DP1/2 may interact weakly with the TIP49 complex. Thus, the E2F1 transactivation domain recruits the same two nuclear cofactors (TRRAP and TIP49) as c-Myc.

TIP49 is essential for E1A-mediated oncogenesis

We showed previously that TRRAP was required for oncogenic transformation by the E1A viral oncoprotein and we were interested to determine if TIP49 might also be involved in this pathway (McMahon *et al.*, 1998). The wtTIP49 and dominant negative TIP49(D302N) were tested in the E1A-dependent transformation of early passage rat embryo fibroblasts in co-operation with the H-ras(G12V) oncogene. Cotransfection of the TIP49(D302N) mutant with E1A and H-ras(G12V) almost completely abolished oncogenic focus formation, whereas cotransfection of wild type TIP49 had only a modest inhibitory effect (Figure 2b). Inhibition of transformation by wild type TIP49 is eliminated at lower concentrations of cotransfected expression vector (data not shown) and is probably due to an unbalanced titration of subunits in multisubunit complexes with the related TIP48 protein. The inhibition of oncogenic activity by TIP49(D302N) is not due to a general toxic effect on cell proliferation because this mutant does not inhibit colony formation with a cotransfected antibiotic selectable marker (Wood *et al.*, 2000). A more thorough examination of potential effects on the cell cycle is presented below.

Dominant inhibition of TIP49, but not TRRAP, promotes E2F1-dependent apoptosis

Overexpression of E2F1 in fibroblasts in the presence of severely limiting growth or survival factors induces

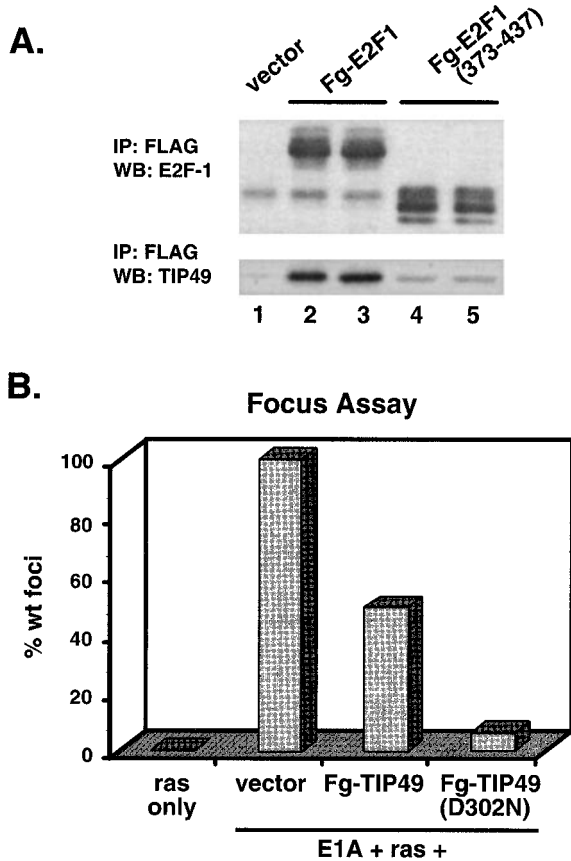


Figure 2 TIP49 is essential for E1A-mediated oncogenesis. (a) 293 cells were transiently transfected with expression vectors for FLAG-tagged full-length E2F1 or FLAG-E2F1Δ373–437. Ly-sates were prepared and E2F1 proteins immunoprecipitated (IP) with anti-FLAG antibody. Precipitated proteins were resolved by SDS–PAGE and Western blotted for either E2F1 or TIP49. Lane 1, IP from cells expressing the FLAG epitope alone. Lanes 2 and 3, IP from cells expressing Fg-E2F1. Lanes 4 and 5, IP from cells expressing Fg-E2F1Δ373–437. (b) Primary rat embryo fibroblast were transfected with expression vectors for E1A, H-RasG12V, TIP49 and TIP49(D302N) in the different combinations indicated. The number of transformed foci is shown on the y-axis. Three plates were assayed for each bar

apoptosis (Qin *et al.*, 1994; Wu and Levine, 1994). To study the activities of different cofactors, we transfected an expression vector for E2F1 with TIP49 or TRRAP along with the membrane-localized GFP to mark the transfected cells. Cells were harvested after 48 h and analysed for apoptosis by the appearance of cells with a sub-G1 DNA content. In the presence of serum, E2F1 did not induce apoptosis and the addition of wild type or dominant inhibitory forms of TIP49 and TRRAP had no effect (data not shown). Starving the cells in 0.1% serum after transfection led to a low level of apoptosis with E2F1 alone (Figure 3a, top right panel). The E2F1 dependence of the apoptosis was established by the lack of cells with a sub-G1 DNA content in the non-GFP (nontransfected) cells (Figure 3a, top left panel) or in GFP-expressing cells transfected with empty vector (data not shown). Supplementing the transfections with wt TIP49 had

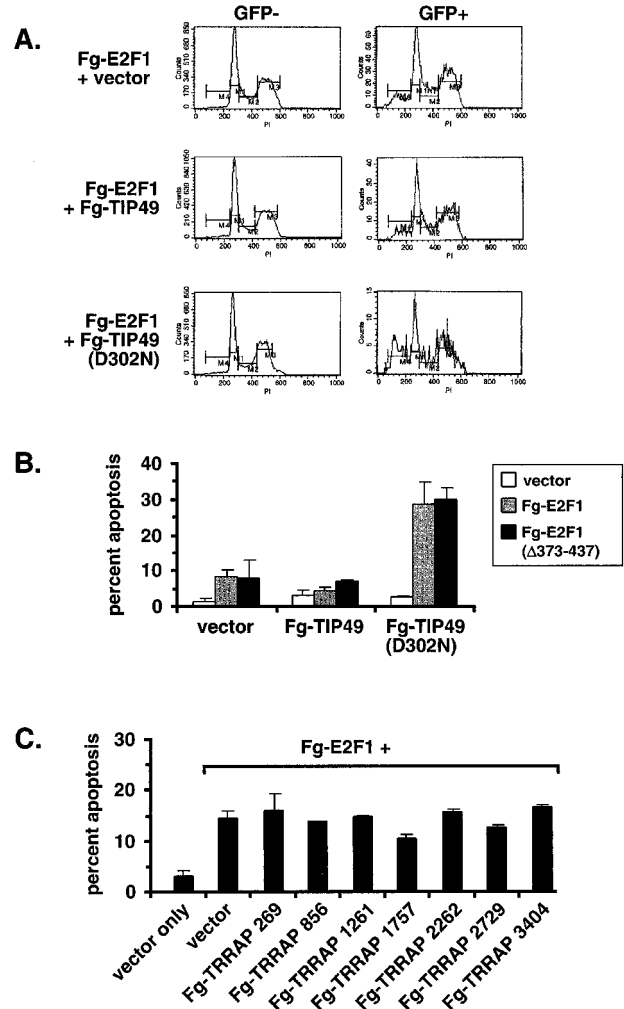


Figure 3 TIP49, but not TRRAP, enhances E2F-1 dependent apoptosis. (a) Representative flow cytometry profiles for TGR cells transfected with the indicated expression constructs following serum starvation (0.1%) for 24 h. DNA content was measured by fluorescence with propidium iodide staining. M1, M2, M3 and M4 represent gating for G1, S, G2/M and sub-G1 populations, respectively. (b) TGR cells were transiently transfected with 5 μg of a vector expressing the FLAG epitope alone, FLAG-TIP49 or FLAG-TIP49D302N in conjunction with vector, FLAG-E2F1 or FLAG-E2F1Δ373–437 and 0.5 μg of a construct expressing GFP. The cells were starved in DMEM with 0.1% FCS. Both floating and adherent cells were harvested and analysed by flow cytometry to determine the percentage of apoptotic cells. The per cent of GFP-positive cells (normalized as in Figure 5b) with a sub-G1 DNA content is plotted on the y-axis. (c) Same experimental procedure as for (b) except cells were transiently transfected with a vector expressing the FLAG epitope alone and various FLAG–TRRAP constructs (5 μg; see Materials and methods) as indicated, in conjunction with vector, FLAG-E2F1 or FLAG-E2F1Δ373–437 and a construct expressing GFP

no effect on E2F1-mediated apoptosis (Figure 3a, middle right panel). In contrast, the dominant negative TIP49(D302N) mutant dramatically enhanced apoptosis with E2F1 (Figure 3a, bottom right panel), with over 29% of the transfected cells undergoing apoptosis (Figure 3b). Thus, disruption of TIP49 activity by overexpressing the ATPase domain mutant greatly

potentiates the apoptotic activity of two different transcription factors.

Unlike the findings for c-Myc in which a mutant in the highly conserved MBII region of the transactivation domain disrupts apoptosis, deletion of the transactivation domain does not impair E2F1-dependent apoptosis (Hsieh *et al.*, 1997). Consistent with this previous mapping, deletion of the transactivation domain had no effect on the low level of apoptosis induced by E2F1 alone, and apoptosis was still dramatically enhanced by the TIP49D302N mutant (Figure 3b). Thus, even though the Myc(Δ 129–145) and E2F1 (Δ 373–437) mutations interfere with the interaction of the respective transactivation domains with TIP49 (Wood *et al.*, 2000 and Figure 1), the apoptotic response differs. This differential response is likely to involve the residual binding to the mutant E2F1 (Figure 2) as well as a complex dependence on other cofactors and cellular pathways. As with c-Myc above, none of the TRRAP expression vectors had any positive or negative effects of E2F1-mediated apoptosis (Figure 3c).

Inhibitory forms of TIP49 and TRRAP do not disrupt cell cycle progression

A third well established biological activity of the c-Myc and E2F1 transcription factors is to promote cell proliferation, primarily in the G1/S phase (Johnson *et al.*, 1993; Mateyak *et al.*, 1997). c-Myc is also required for G2/M since c-myc null cells have a prolonged G2/M phase as well as an extended G1 (Mateyak *et al.*, 1997). While the MBII domain is required for efficient cell cycle progression, c-MycMBII Δ can still rescue a large fraction of the growth defect in myc-null cells (Bush *et al.*, 1998; Nikiforov *et al.*, 2002). The c-myc-null cells have a cell doubling time of approximately 50 h, compared to a doubling time of 18 h for the parental myc diploid or c-Myc reconstituted cells. Reconstitution with the c-MycMBII Δ mutant restores the doubling time to 33 h, and reconstitution with N-MycMBII Δ restores the doubling time to 26 h (Bush *et al.*, 1998; Nikiforov *et al.*, 2002). These observations argue that the cofactors that bind to Myc through MBII are not essential for cell proliferation, but we were interested to test individual cofactors in a more direct fashion. Rat1 fibroblast lines that stably express TIP49 and TIP49(D302N) were analysed for the cell cycle distribution in both high and low serum concentrations. There was no significant increase in the fraction of cells in G1 plus G2/M with either wild type or dominant negative forms of TIP49, regardless of serum concentrations (Figure 4a,b). Growth curves for cells in 10% serum confirm the lack of any defect in cell cycle progression (Figure 4c). Transient transfection of the same TIP49 vectors with GFP to mark transfected cells also had no impact on cell cycle distribution (Figure 3a). Similarly, transient expression of TRRAP segments had no effect on cell cycle distribution (Figure 4d,e), even for segments that inhibit oncogenic transformation. These findings suggest that the binding of Myc to TIP49 and TRRAP

cofactors complexes is not rate-limiting for normal cell growth.

Discussion

Both c-Myc and E2F1 interact with diverse cofactors that mediate their role in oncogenesis, cell proliferation and apoptosis. Previous work showed that both of these transcription factor families bind to TRRAP (McMahon *et al.*, 1998), an essential component of the SAGA chromatin modifying complex. Recent work shows that TRRAP mediates at least part of the transcriptional activation by both families (Bouchard *et al.*, 2001; Frank *et al.*, 2001; Lang *et al.*, 2001). We show in this study that the overlap in nuclear cofactors shared by Myc and E2F1 extends to TIP49 and the recruitment of distinct cofactors allowed us to initiate a functional dissection of cofactor activities. Both c-Myc and E2F1 can function as either oncogenes or inducers of apoptosis, depending on the complex signals that determine the cellular responses to overexpression of these transcription factors. The regulatory signals and downstream effectors for these divergent activities are of great interest for understanding the outgrowth of tumors. The TRRAP protein appears to be predominantly required for oncogenic transformation by c-Myc and E2F1, since dominant inhibitory expression vectors of TRRAP block focus formation in cooperation with the H-rasG12V oncogene (McMahon *et al.*, 1998). Expression of the same vectors has no effect on apoptosis induced by either c-Myc or E2F1, nor do they induce a G1 arrest in normal proliferating cells. This segregation of TRRAP activity to oncogenesis is supported by structure/function analysis of the transcription factors themselves. The MycS protein is a naturally occurring form of c-Myc that initiates at methionines just N-terminal to the conserved MBII domain (Xiao *et al.*, 1998). MycS can rescue Myc-dependent cell proliferation and induce apoptosis (Xiao *et al.*, 1998), but MycS cannot cooperate with H-rasG12V in oncogenic transformation or bind to TRRAP (McMahon *et al.*, 1998). Similarly, a C-terminal deletion of E2F1 disrupts oncogenic activity (Singh *et al.*, 1994) and TRRAP binding (McMahon *et al.*, 1998) but not the induction of apoptosis (Hsieh *et al.*, 1997). Finally, deletion of MBII from N-Myc abolishes oncogenic transformation in cooperation with an H-ras oncogene but largely rescues the growth defect of c-myc-null cells (Nikiforov *et al.*, 2002). Thus, the recruitment of TRRAP (and possibly hGCN5 and/or PCAF histone acetyltransferase activities) appear to be a critical rate-limited step in oncogenic transformation but not in other c-Myc and E2F1 activities.

The TIP49 protein appears to be a key modulator of apoptotic activity for both c-Myc and E2F1. Since both the induction of apoptosis and binding to the TIP49 complex is dependent on the conserved MBII domain of c-Myc, we initially expected that a dominant inhibitor of cofactor activity might suppress apoptosis. We were surprised to find just the opposite, that the

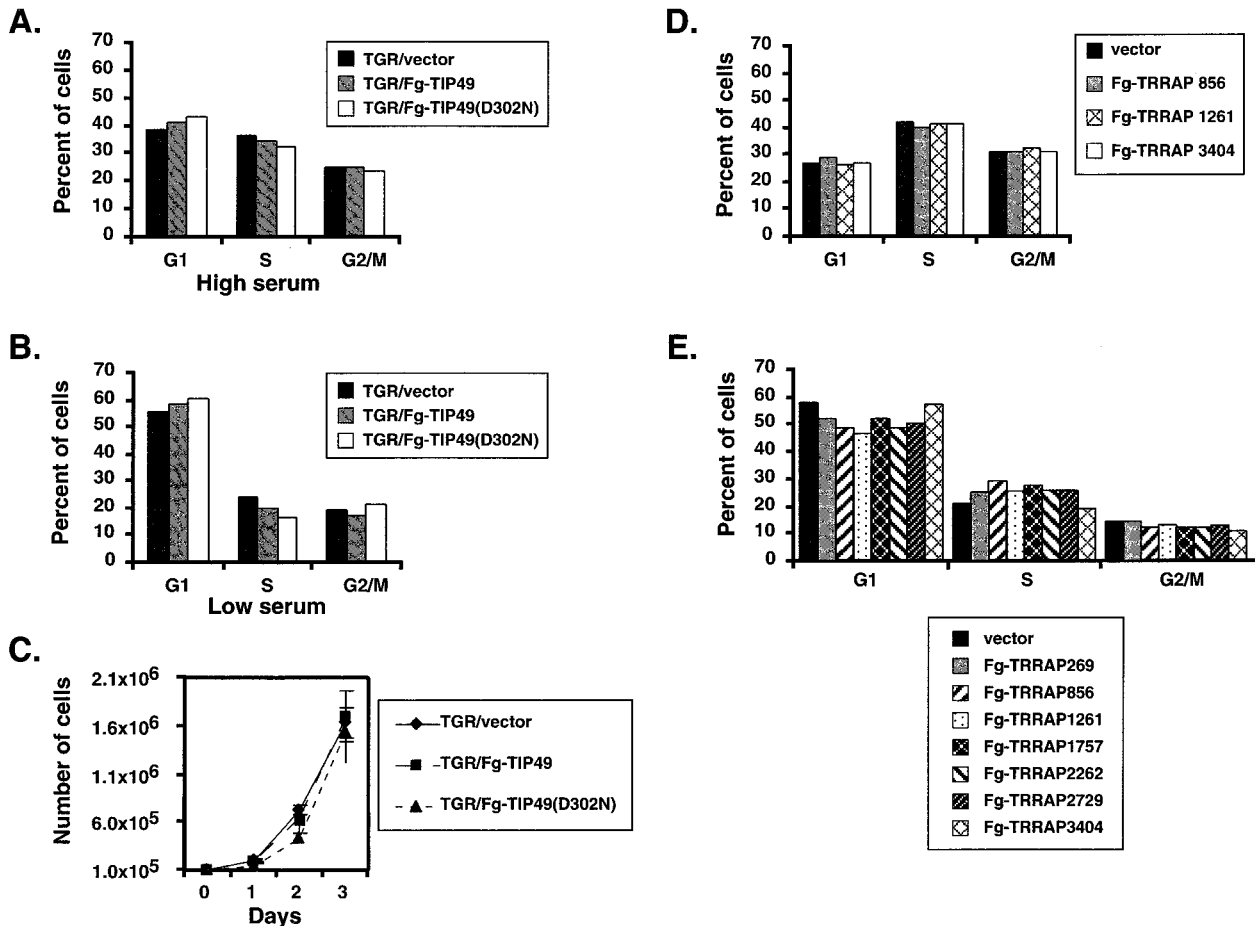


Figure 4 Dominant negative TIP49 and TRRAP vectors do not disrupt cell cycle progression (a) Rat1 fibroblast lines were created to stably express the FLAG epitope alone (vector), FLAG-TIP49 or FLAG-TIP49(D302N). Log phase growing cells were harvested and cell cycle distribution determined by flow cytometry using propidium iodide staining of the DNA. (b) The same stable cell lines used in (a) were starved in DMEM with 0.1% FCS. Both floating and adherent cells were harvested. Cell cycle distribution was determined by flow cytometry as in (a). (c) Growth curves for the stable cell lines used in (a) and (b) demonstrate equivalent doubling times. (d) Rat1 cells were transiently transfected with 5 μ g of a vector expressing the FLAG epitope alone and the various FLAG-TRRAP constructs (see Materials and methods) indicated, plus 0.5 μ g of a construct expressing GFP. Log phase growing cells were harvested and cell cycle distribution determined as in (a). (e) Rat1 cells were transiently transfected with 5 μ g of the indicated expression vectors plus 0.5 μ g of a construct expressing GFP. The cells were starved in DMEM with 0.1% FCS. Both floating and adherent cells were harvested. Cell cycle distribution was determined by flow cytometry as in (a)

TIP49(D302N) mutant enhanced apoptosis while blocking oncogenic transformation in parallel assays. One simple resolution of these findings might be that the suppression of oncogenic transformation is directly tied to the induction of apoptosis. Primary rat embryo cells undergoing Myc-dependent or E1A-dependent oncogenic transformation may be sensitized to apoptosis induced by TIP49(D302N) through the same mechanism that sensitizes rat fibroblasts overexpressing c-Myc, E2F-1, or E1A alone (Evan *et al.*, 1995; White, 1995; Wu and Levine, 1994). It is also noteworthy that E2F1 has recently been reported to be required for Myc-mediated apoptosis (Leone *et al.*, 2001), suggesting that the dominant negative TIP49(D302N) mutant could be affecting both transcription factor networks simultaneously in our assays.

Why would the disruption of a critical cofactor activity induce apoptosis? There are at least two

models that could account for this finding. Different cofactor complexes may be critical for distinct subsets of target genes, and the disruption of one complex could suppress anti-apoptotic gene expression and/or enhance pro-apoptotic gene expression. There is no clear consensus for the target genes that promote c-Myc or E2F1-dependent apoptosis, but E1A-mediated apoptosis has been linked to the suppression of the p300-dependent mdm2 negative-feedback loop that inhibits the accumulation of p53 protein (Thomas and White, 1998). On the other hand, E2F1 can induce apoptosis in cells that are genetically deficient for both Rb and p53, indicating that p53 is not an essential effector (Hsieh *et al.*, 1997). p19ARF is induced by both E2F1 and Myc (Nevins, 1995; Sherr, 1998; Stanchina *et al.*, 1998; Zindy *et al.*, 1998), but equivalent induction was observed with non-apoptotic E2F2 (DeGregori *et al.*, 1997). Gene expression profiles

associated with the overexpression of TIP49(D302N) would be very valuable in identifying key target genes in the c-Myc or E2F1 apoptotic pathways.

An alternate model for the enhancement of apoptosis by TIP49(D302N) is the induction of a direct apoptotic signal by recruitment of defective ATPase/helicase complexes to genomic sites. Disruption of helicase-related activity could either cause genomic damage that potentiates an apoptotic cascade or lead to a general failure to repair genomic damage with similar consequences. In the latter model, no specific direct target genes would necessarily be implicated in enhanced apoptosis. A recent report suggests that the TIP48/TIP49 ATPase/helicase-related protein are part of a complex with the TIP60 histone acetylase and that acetylase-defective TIP60 can suppress apoptosis in HeLa cells (Ikura *et al.*, 2000). However, we find no significant TIP60 in our c-Myc or TIP49 immunoprecipitates either *in vivo* or *in vitro* (Park *et al.*, 2002). The yeast homologs of TIP49 and TIP48 (called Rvb1p and Rvb2p) were recently found in a chromatin remodeling complex containing the Swi2/Snf2-related Ino80p, and loss of Ino80 causes a sensitivity to DNA damaging agents (Ebbert *et al.*, 1999; Shen *et al.*, 2000). No similar complex has been described in mammalian cells. Finally, TIP48 and TIP49 have also been found in a complex with the E1A-associated p400 protein (Fuchs *et al.*, 2001), although we find the majority of mammalian TIP49 is in a complex that is distinct from those associated with TRRAP (G LeRoy and G Wang, unpublished observations). One explanation for these diverse findings is that the TIP48/TIP49 ATPase functions in several different complexes involved in transcription and/or genomic integrity, and disruption of their function has different effects dependent on the cellular context.

In contrast to oncogenic transformation and apoptosis, the cofactors analysed in this study do not appear to have a readily assayed role in normal cell proliferation. The MBII domain of c-Myc that is required for TRRAP and TIP49 binding also contributes to c-Myc function in fibroblast proliferation, but cells reconstituted with Myc Δ MBII grow faster than c-myc-null cells, implying that Myc has functions outside of the factors recruited through MBII (Bush *et al.*, 1998; Nikiforov *et al.*, 2002). It is possible that the proliferation of normal cells is dependent on other cofactors, which for E2F1 could include p300 or a repressor activity recruited by Rb. However, another simple explanation is that only low levels of cofactor activity are required in proliferating cells, where it has been estimated that 450 molecules of c-Myc per cell are sufficient for progression through the cell cycle (Mehmet *et al.*, 1997). The dominant inhibitory vectors do not create a complete loss of cofactor function since the wild type cofactor is still expressed. Genetic studies in yeast demonstrate that TRRAP (Tra1p) and TIP49 (Rvb1) are essential for viability, and a complete loss of TRRAP function is nonviable in mammalian cells (Herceg *et al.*, 2001; Saleh *et al.*, 1998; Wood *et al.*,

2000). Since neither Myc nor E2F exists in yeast, the nonviability of mammalian cells with a TRRAP knockout is unlikely to be due exclusively to defects in the Myc and E2F pathways. Temperature sensitive alleles of Rvb2(Tih2) causes a rapid G1 arrest and suppression of specific gene expression at the nonpermissive temperature, emphasizing the requirement for these ATPase/helicase proteins in transcription and cell cycle progression (Lim *et al.*, 2000).

A primary function of most, if not all, sequence-specific transcription factors is to recruit nuclear complexes to chromosomal sites. Many of the complexes involved in chromatin remodeling have enzymatic functions such as HAT, HDAC and ATPase activities. This study shows that the disruption of specific cofactors can have selected effects on oncogenesis and apoptosis induced by Myc and E2F1, but that normal cell proliferation is relatively insensitive under comparable conditions. Since these complexes are required to modulate the oncogenic and apoptotic activities of transcription factors like Myc and E2F, the associated enzyme activities are potential therapeutic targets in cancer cells.

Materials and methods

Transfection and immunoprecipitation

HEK293 cells were cultured in Dulbecco's modified Eagle's media (DMEM), supplemented with 10% fetal calf serum (GIBCO-BRL). Cells were transfected with 2–4 μ g of each indicated (see figure legends) expression vector using the calcium phosphate method and lysed using F buffer (Sommer *et al.*, 1998). For immunoprecipitations, lysates were incubated with anti-FLAG antibodies in conjunction with protein G beads. Precipitates were then analysed by Western blotting with anti-FLAG or anti-TIP49 (Wood *et al.*, 2000). Protein expression was determined by analysing the lysates by Western blotting with the appropriate antibody. Antibody detection was performed using ECL (Amersham).

Transformation experiments

Rat embryo fibroblast transformation assays were performed as previously described (Wood *et al.*, 2000). Transfections included H-rasG12V (1 μ g) and a CMV promoter driven E1A expression vector (1 μ g) supplemented with either FLAG epitope alone (2 μ g), FLAG-TIP49 (2 μ g) or FLAG-TIP49(D302N) (2 μ g). Transfections were performed in triplicate using the calcium phosphate method.

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Cells were transfected with 0.5 μ g pBB14(GFP) and various expression vectors, as indicated in the figure legends, and refed with DMEM plus 0.1% calf serum to induce apoptosis. Floating and adherent cells were harvested after 20–24 h of starvation. The cells were pelleted and the pellet resuspended in 500 μ l PBS+0.5% fetal calf serum and fixed in 70% ethanol at -20° C overnight. The fixed cells were pelleted and the pellet resuspended in 500 μ l PBS+0.5% fetal calf serum. Five hundred μ l of DNA extraction buffer (192 ml, 0.2 M Na₂HPO₄ with 8 ml 0.1 M citric acid – final pH 7.8) was added to each sample followed by incubation for 5 min at

room temperature. The cells were pelleted and resuspended in 800 μ l PBS + 0.5% fetal calf serum supplemented with 8 μ l propidium iodide (1 mg/ml in 20 mM sodium citrate) and 4 μ l RNase A (DNase free, 10 mg/ml) in 1 ml total volume. The cells were incubated for 30 min at 37°C and analysed on a Becton Dickinson FACScan Fluorescence Activated Cells Sorter.

TRRAP constructs

All TRRAP fragments were expressed from a CMV promoter-driven FLAG epitope-containing vector. Fg-TRRAP269 corresponds to amino acids 269–598 of TRRAP; Fg-TRRAP856, corresponds to aa 856–1134; Fg-TRRAP81261, corresponds to aa 1261–1579; Fg-

TRRAP1757, corresponds to aa 1757–2260; Fg-TRRAP2262, corresponds to aa 2262–2729; Fg-TRRAP2729, corresponds to aa 2729–3330; and Fg-TRRAP3404, corresponds to aa 3404–3830. All other constructs are described in figure legends.

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