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Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc

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The human proto-oncogene c-*myc* encodes a highly unstable transcription factor that promotes cell proliferation. Although the extreme instability of Myc plays an important role in preventing its accumulation in normal cells, little is known about how Myc is targeted for rapid destruction. Here, we have investigated mechanisms regulating the stability of Myc. We show that Myc is destroyed by ubiquitin-mediated proteolysis, and define two elements in Myc that oppositely regulate its stability: a transcriptional activation domain that promotes Myc destruction, and a region required for association with the POZ domain protein Miz-1 that stabilizes Myc. We also show that Myc is stabilized by cancer-associated and transforming mutations within its transcriptional activation domain. Our data reveal a complex network of interactions regulating Myc destruction, and imply that enhanced protein stability contributes to oncogenic transformation by mutant Myc proteins.

Keywords: Miz-1/Myc/transcription/ubiquitin-mediated proteolysis

Introduction

The c-*myc* proto-oncogene encodes a basic helix–loop–helix transcription factor that features prominently in the regulation of cell proliferation (reviewed by Henriksson and Luscher, 1996). Through association with its partner protein Max, Myc binds to promoter elements in, and regulates the expression of genes required for cell cycle progression (e.g. the cdc25 phosphatases; Galaktionov *et al*., 1996) and cellular proliferation (e.g. ornithine decarboxylase; Bello-Fernandez *et al*., 1993). Consistent with its normal role in promoting cell growth, Myc can drive oncogenic transformation, and deregulated Myc expression is associated with a variety of human cancers (for review see Spencer and Groudine, 1991).

The expression and activity of Myc are tightly regulated at many levels, including transcription, mRNA stability, translation and protein stability (Spencer and Groudine, 1991). Indeed, Myc is a highly unstable protein, with a typical half-life of 30 min (Hann and Eisenman, 1984). The extreme instability of Myc is typical of many of the transcription factors that regulate cell growth: for example, p53 (Mora *et al*., 1982; Oren *et al*., 1982; Hann and Eisenman, 1984), Jun (Treier *et al*., 1994), Fos (Curran *et al*., 1984) and E2F (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Campanero and Flemington, 1997) are all unstable proteins, with *in vivo* half-lives ranging from a few minutes to a few hours. The rapid and controlled destruction of these proteins keeps their intracellular levels responsive to environmental factors, such as mitogenic signals (Musti *et al*., 1997), and maintains tight control over cell proliferation, a role which is underscored by the fact that some oncogenic forms of these transcription factors escape degradation (Papavassiliou *et al*., 1992; Treier *et al*., 1994; Tsurumi *et al*., 1995) and accumulate to higher levels than their wild-type counterparts.

The majority of short-lived transcription factors are destroyed by ubiquitin-mediated proteolysis, a process in which covalent attachment of the protein ubiquitin (Ub) signals transcription factor destruction by the 26S proteasome (for review see Varshavsky, 1997). Protein ubiquitylation is a highly specific multistep process, which begins when an element within the target protein, termed a 'degron', is recognized by a Ub-conjugating enzyme (Ubc, or E2), either alone, or in conjunction with a Ub–protein ligase (Ubl, or E3). After the degron has been bound and recognized, Ub is then transferred to a lysine residue within the target protein. Repeated rounds of ubiquitylation result in a highly ubiquitylated target protein which is destroyed rapidly by the proteasome. Because proteasomal destruction depends on substrate ubiquitylation, selective degron recognition is central to the control of Ub-mediated proteolysis.

Myc has been shown to be destroyed by Ub-mediated proteolysis (Ciechanover *et al*., 1991; Flinn *et al*., 1998; Gross-Mesilaty *et al*., 1998), but regions of Myc that regulate its destruction have not been characterized. Here, we have studied how Myc is targeted for proteolysis. We find that Myc destruction is governed by the action of two opposing elements, a transcriptional activation domain that promotes Myc destruction, and a region required for association with Miz-1 that stabilizes Myc. We show that Myc destruction occurs in yeast and in mammalian cells, and demonstrate that transforming and cancer-associated mutations stabilize Myc. Our data suggest that Myc destruction is a highly regulated process that plays an important role in the control of cellular proliferation and in cancer.

Results

Myc is destroyed by ubiquitin-mediated proteolysis in vivo

The c-Myc protein has been shown to be destroyed by Ub-mediated proteolysis *in vitro* (Ciechanover *et al*., 1991; Gross-Mesilaty *et al*., 1998), and its destruction is blocked

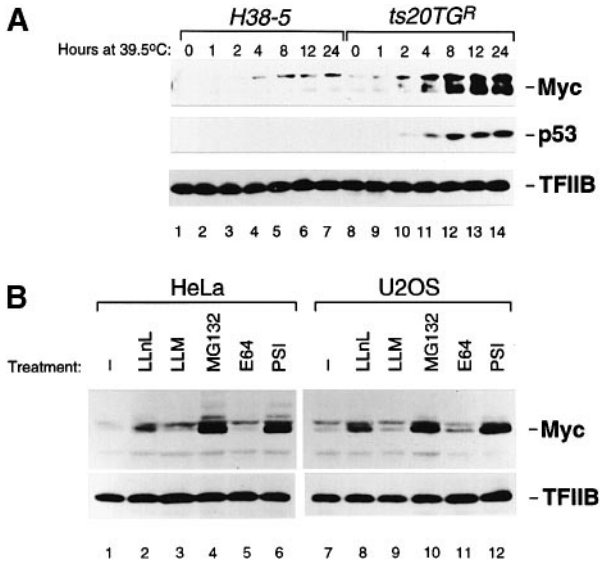

Fig. 1. A functional Ub–proteasome pathway is required for low steady-state levels of Myc *in vivo*. (**A**) Myc accumulates in mouse cells with a temperature-sensitive ubiquitin-activating enzyme. Mouse *H38-5* (lanes 1–7) and *ts20TG*^{R} (lanes 8–14) cells, grown at the permissive temperature of 35°C, were shifted to the restrictive temperature of 39.5°C for the times indicated, and cell extracts prepared as described in Materials and methods. Extracts were then resolved by SDS–PAGE, and Myc, p53 and TFIIB levels determined by immunoblotting. (**B**) Proteasomal inhibition causes Myc accumulation. Human HeLa (lanes 1–6) and U2OS (lanes 7–12) cells were treated with either DMSO solvent ('–'; lanes 1 and 7), or with 25 μM each of LLnL, LLM, MG132, E64 or PSI, dissolved in DMSO. Treatment was for 12 h, after which cell extracts were prepared, and Myc and TFIIB levels determined by SDS–PAGE and immunoblotting.

by proteasomal inhibition *in vivo* (Gross-Mesilaty *et al*., 1998). To gain more insight into how Myc is normally destroyed *in vivo*, we sought additional evidence implicating the involvement of the Ub–proteasome pathway in Myc destruction.

We first asked whether a functional Ub-activating enzyme (Uba, or E1), an enzyme required for all Ub conjugation (Varshavsky, 1997), is required for Myc turnover *in vivo*. Chowdary *et al*. (1994) have described a mouse cell line, *ts20TG*^{R}, with a temperature-sensitive defect in the single mouse E1. We examined Myc levels in *ts20TG*^{R} cells grown at the restrictive temperature (39.5°C), and in similarly treated *H38-5* cells, in which the temperature-sensitive defect has been compensated by introduction of sequences encoding wild-type human E1. Results of this analysis are shown in Figure 1A. In both *H38-5* and *ts20TG*^{R} cells, steady-state levels of Myc were low prior to shifting growth temperature (lanes 1 and 8). Shifting *H38-5* cells to the restrictive temperature had little effect on the steady-state levels of Myc (lanes 1–7). Shifting *ts20TG*^{R} cells to the restrictive temperature, however, produced a large increase in Myc levels (compare lane 8 with lanes 9–14). A similar pattern of induction was observed with p53, in agreement with Chowdary *et al*. (1994), but not with the basal factor TFIIB. The accumulation of Myc in *ts20TG*^{R} cells grown at 39.5°C demonstrates that Ub activation is required for the low steady-state levels of Myc normally found in these cells.

We next examined the effects of proteasome inhibition on Myc accumulation in two human cell lines, the cervical carcinoma cell line HeLa and the osteosarcoma cell line U2OS. The results of this experiment are shown in Figure 1B. In the absence of inhibitors, Myc levels were low (lanes 1 and 7). Treatment of both cell lines with the proteasome inhibitors LLnL (Rock *et al*., 1994; lanes 2 and 8), MG132 (Rock *et al*., 1994; lanes 4 and 10) and PSI (Traenckner *et al*., 1994; lanes 6 and 12) produced a marked increase in Myc accumulation. This effect was specific to proteasomal inhibitors; treatment of cells with the calpain inhibitor LLM (Rock *et al*., 1994; lanes 3 and 9) or the lysosomal protease inhibitor E64 (lanes 5 and 11) produced no change in Myc accumulation. These results demonstrate that the proteasome is required for the low steady-state level of Myc in these cells. Similar results were obtained with inhibitor treatment of human IMR-90 and rat REF-52 diploid fibroblasts (data not shown).

We have been unable to measure the stability of endogenous Myc in either HeLa or U2OS cells following proteasome inhibition. Therefore, to determine whether proteasome inhibition directly stabilizes the Myc protein, we transiently expressed epitope-tagged Myc in HeLa cells and measured its stability using pulse–chase analysis. The results of this analysis are shown in Figure 2A. In the absence of proteasome inhibitor ('–MG132'), transiently expressed Myc was unstable, with an estimated half-life of ~35 min. If, however, cells were treated with the proteasome inhibitor MG132 for 2 h prior to the pulse–chase analysis ('+MG132'), the half-life of Myc was increased to ~150 min, demonstrating that rapid Myc destruction in these cells requires the proteasome.

Finally, we asked whether Myc is ubiquitylated *in vivo*. To do this, we tested whether hemagglutinin (HA) epitope-tagged Myc could become covalently linked to polyhistidine-tagged human ubiquitin (His-Ub; Treier *et al*., 1994) in a transient co-transfection assay, the results of which are shown in Figure 2B. As a control, we compared Myc ubiquitylation with that of another transcription factor, Jun. As expected (Treier *et al*., 1994), Jun displayed a high level of ubiquitylation in this assay (lane 4). Similarly, Myc also displayed a high level of ubiquitylation, as revealed by the presence of a smear of HA-reactive material that carried both the HA- and His-Ub tags (lane 6). This material was only observed in the presence of His-Ub ubiquitin (compare lanes 5 and 6) and was larger than free HA-tagged Myc by at least 9 kDa (data not shown), corresponding to the attachment of multiple His-Ub groups. Thus, like Jun, Myc is ubiquitylated *in vivo*. Although Myc appears to be less heavily ubiquitylated than Jun in this assay, this difference is unlikely to be meaningful. To be detected in this assay, a protein must not only be ubiquitylated, but it must also be stable enough to be captured in this state. Thus comparison between proteins with different half-lives, i.e. Myc (35 min) and Jun (90 min; Treier *et al*., 1994), does not accurately reflect the relative differences in the propensity of each protein to be ubiquitylated.

The results presented above confirm and extend previous studies (Ciechanover *et al*., 1991; Gross-Mesilaty *et al*., 1998), and argue strongly that Myc is unstable because it is destroyed by Ub-mediated proteolysis.

Positive and negative elements regulate Myc destruction

We next asked which regions of Myc are required for its destruction by Ub-mediated proteolysis. To do this, we

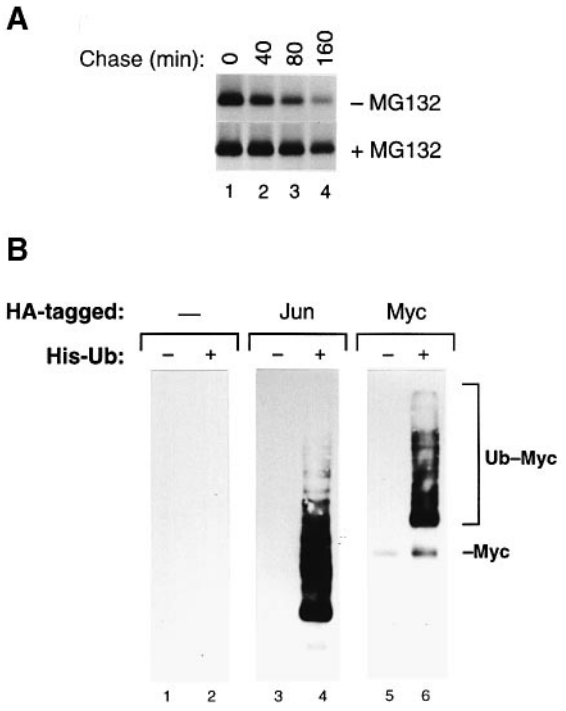

Fig. 2. Destruction of Myc by ubiquitin-mediated proteolysis. (**A**) Proteasome inhibition stabilizes Myc. Human HeLa cells transiently expressing epitope-tagged Myc were treated with either DMSO (–MG132) or 50 μM MG132 (+MG132) for 2 h prior to pulse–chase analysis, as described in Materials and methods. Denaturing immunoprecipitation was used to recover labeled Myc proteins, which were resolved by SDS–PAGE and visualized by autoradiography. (**B**) Myc is ubiquitylated *in vivo*. HeLa cells were transiently transfected with expression constructs encoding HA epitope-tagged Jun (lanes 3 and 4) or Myc (lanes 5 and 6) either in the presence (even-numbered lanes) or absence (odd-numbered lanes) of an expression construct encoding polyhistidine-tagged Ub (His-Ub). Following transfection, histidine-tagged proteins were purified using nickel affinity chromatography, resolved by SDS–PAGE and HA-tagged proteins detected by immunoblotting. 'Ub-Myc' indicates the multiple ubiquitylated forms of Myc detected in lane 6; '–Myc' indicates the position of free Myc. Approximately 1–2% of Myc can be captured in this ubiquitylated form (data not shown).

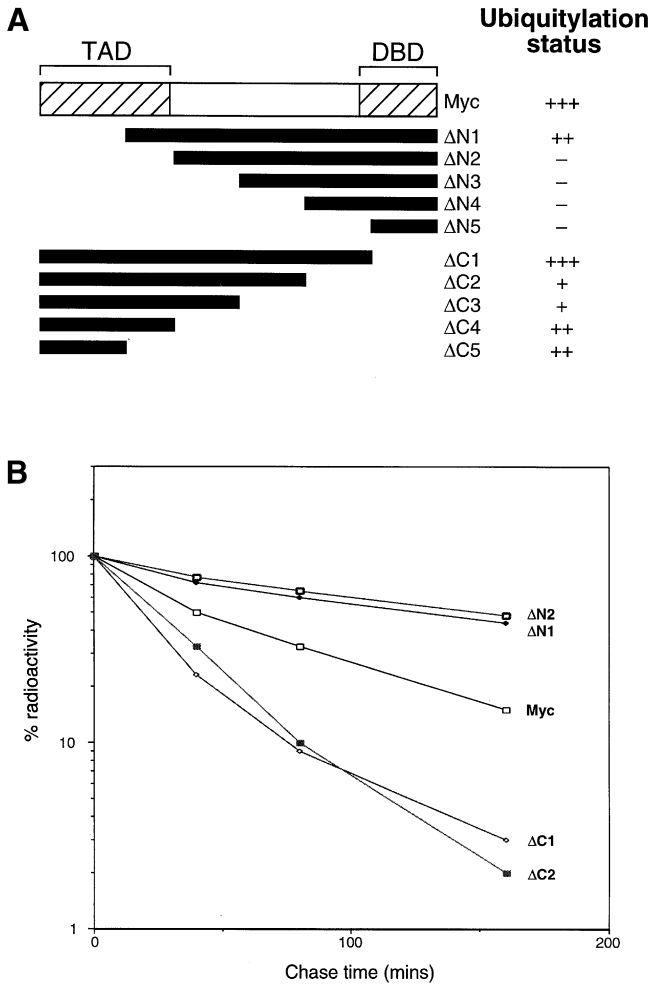

Fig. 3. Two regions of Myc determine its stability. (**A**) Design of N- and C-terminal Myc deletion mutants and their ubiquitylation status in HeLa cells. Myc deletion mutants were generated to remove residues encoding Myc sequences 2–94 (ΔN1), 2–147 (ΔN2), 2–220 (ΔN3), 2–293 (ΔN4) and 2–366 (ΔN5), or 367–439 (ΔC1), 294–439 (ΔC2), 221–439 (ΔC3), 148–439 (ΔC4) and 95–439 (ΔC5). Ubiquitylation status was determined by transient transfection analysis in HeLa cells as described in Materials and methods and in the legend to Figure 2. '+++' refers to wild-type Myc ubiquitylation levels; '++' to ~50% wild-type Myc ubiquitylation levels; '+' to ~25% wild-type Myc ubiquitylation levels; and '–' to no detectable ubiquitylation. (**B**) Stability of select Myc proteins in HeLa cells. The indicated Myc mutants were expressed in HeLa cells, and their stabilities determined by pulse–chase analysis as described in Materials and methods. Phosphoimaging was used to determine the percentage of radioactivity present for each Myc species at each time point.

constructed a panel of Myc deletion mutants, depicted in Figure 3A. We transiently expressed these deletion mutants in HeLa cells and measured their ubiquitylation status and, in certain instances, their stability. This analysis is summarized in Figure 3.

Deletion of the first 94 residues of Myc (ΔN1) modestly reduced Myc ubiquitylation (Figure 3A, ΔN1) and stabilized Myc considerably (Figure 3B, ΔN1), increasing its half-life to ~140 min. Further deletion of N-terminal residues to position 147 (ΔN2) abrogated detectable ubiquitylation (Figure 3A, ΔN2), although the half-life of Myc was not greatly increased above that of the ΔN1 mutant (Figure 3B, compare ΔN1 and ΔN2). Because the ΔN2 deletion blocked Myc ubiquitylation and stabilized Myc to the same extent as proteasome inhibition (Figure 2A), we conclude that sequences within the first 147 amino acids of Myc are required for its destruction by Ub-mediated proteolysis. In contrast, none of the C-terminal deletions abrogated Myc ubiquitylation (Figure 3A, ΔC1–ΔC5). However, these deletions did affect Myc stability considerably: deletion of Myc residues 367–439 (ΔC1) markedly destabilized the protein, reducing the half-life of Myc to ~20 min (Figure 3B, ΔC1). Further deletion of C-terminal sequences (ΔC2; 294–439) did not destabilize Myc further (Figure 3B, compare ΔC1 and ΔC2). The destabilization of Myc by deletion of residues 367–439 argues that this region normally acts to enhance Myc stability.

Taken together, these results suggest that Myc stability is governed by the action of two opposing elements: an N-terminal element that promotes Myc destruction and a C-terminal element that stabilizes Myc. To characterize these two elements further, we asked if they could modulate the stability of a heterologous protein sequence, in this case the DNA-binding domain (DBD) of the yeast transcription

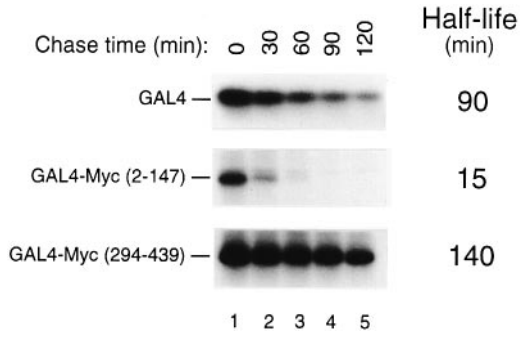

Fig. 4. The elements which regulate Myc stability can regulate stability of a heterologous protein sequence. Human HeLa cells were transiently transfected with expression constructs encoding either the GAL4 DBD alone ('GAL4'), or the GAL4 DBD fused to Myc residues 2–147 or 294–439. Protein stability was determined by pulse–chase analysis as described in Materials and methods. The calculated half-life for each GAL4 protein is shown.

factor GAL4. Sequences corresponding to residues 2–147 and 294–439 of Myc were fused to the GAL4 DBD, the chimeric proteins expressed in HeLa cells, and their stabilities determined by pulse–chase analysis, as shown in Figure 4. The GAL4 DBD alone was moderately stable, with a half-life of ~90 min. Addition of Myc residues 2–147 destabilized the GAL4 DBD considerably, reducing its half-life to ~15 min. In contrast, addition of C-terminal Myc residues 294–439 to the GAL4 DBD stabilized the protein, increasing its half-life to ~140 min. Thus, it appears that sequences within residues 2–147 of Myc are necessary and sufficient to signal protein instability, while sequences within residues 294–439 of Myc encompass a self-contained protein-stabilizing element.

The Myc degron overlaps the Myc TAD

Our data have revealed that sequences 2–147 of Myc signal Ub-mediated destruction, i.e. they function as a degron. Curiously, this region of Myc also encompasses its transcriptional activation domain (TAD; residues 1–143; Kato *et al*., 1990). To define further the Myc degron and to determine whether we could separate transcriptional activation from degron function, we performed a finer deletion analysis on the Myc TAD. A series of N- and C-terminal Myc TAD fragments, depicted in Figure 5A, were fused to the GAL4 DBD and assayed for ubiquitylation status (data not shown), stability, transcriptional activation and steady-state protein accumulation after normalization for RNA expression. This analysis is shown in Figure 5.

All GAL4–Myc TAD chimeric proteins were heavily ubiquitylated, much more so than the GAL4 DBD alone (data not shown). Similarly, all GAL4–Myc TAD proteins activated transcription more than the GAL4 DBD alone (Figure 5B, compare lane 1 with lanes 2–8), and all were less stable than the GAL4 DBD, as revealed by the reduction in their half-lives (Figure 5A) and steady-state levels of accumulation (Figure 5B). Thus, individual regions within the Myc TAD can signal ubiquitylation, activate transcription and confer protein instability. Moreover, there was a good correlation between the ability of these regions to activate transcription and to confer protein instability. For example, residues 2–128 of Myc destabilize

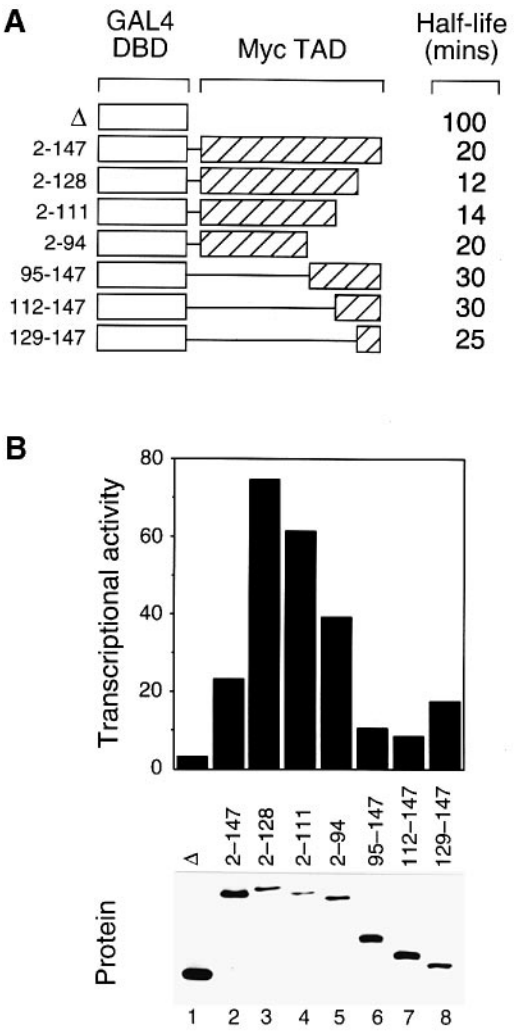

Fig. 5. Multiple regions within the Myc activation domain can activate transcription and signal protein instability. (**A**) Design of GAL4–Myc fusion proteins and their relative stabilities. The indicated residues of Myc were fused in-frame to the GAL4 DBD, transiently expressed in HeLa cells, and protein half-life determined by pulse–chase analysis as described in Materials and methods. The calculated half-life for each GAL4 protein is shown. (**B**) Multiple regions within Myc residues 2–147 can activate transcription and destabilize the GAL4 DBD. Upper panel: expression constructs encoding the indicated GAL4–Myc chimeras were transfected into HeLa cells and transcriptional activation determined as described in Materials and methods. Lower panel: expression constructs encoding the indicated GAL4–Myc chimeras were transfected into HeLa cells. Following transfection, cells were collected, and RNA and protein prepared as described in Materials and methods. Protein samples were then normalized for differences in RNA levels; the immunoblot shown thus represents the steady-state levels of protein for a given amount of RNA.

the GAL4 DBD more than residues 2–147 (reducing the protein half-life to 12 min, compared with 20 min for the 2–147 fragment), are transcriptionally more potent than residues 2–147 (Figure 5B, compare lanes 2 and 3) and reduce protein accumulation to a greater extent. Similar correlations between protein stability, transcriptional activation potency and steady-state accumulation are observed with all other regions of the Myc TAD (lanes 3–8). The ability of separate regions of Myc to activate transcription and to confer protein instability suggests that the Myc degron, like the Myc TAD, is dispersed throughout

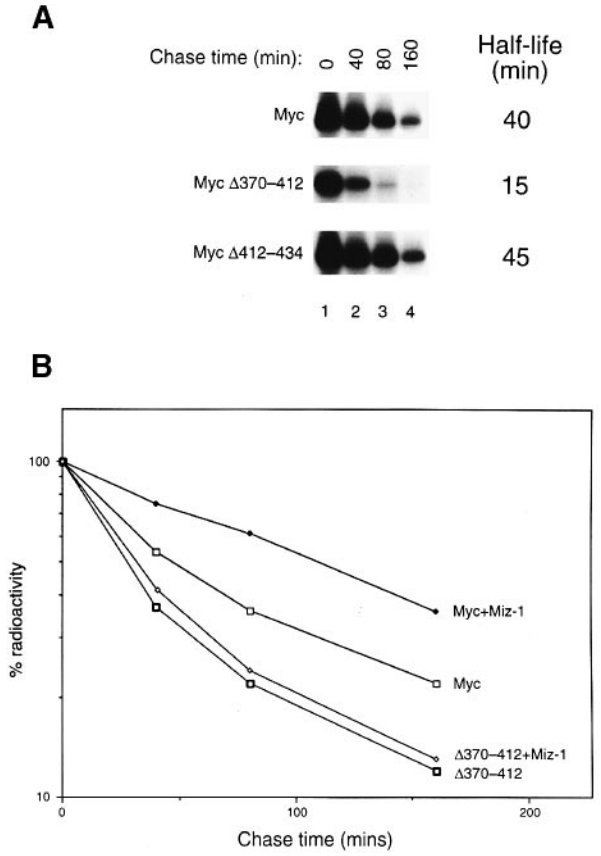

Fig. 6. Miz-1 stabilizes Myc. (**A**) Deletion of Myc sequences required for association with Miz-1 destabilize Myc. Comparison of the stabilities of wild-type Myc and the Myc Δ370–412 and Δ412–434 mutants in HeLa cells. Protein stability was determined by pulse–chase analysis as described in Materials and methods. The half-life of each protein is presented. (**B**) Co-expression of Miz-1 increases Myc stability. HeLa cells were transiently co-transfected with expression constructs encoding full-length Myc, or the Δ370–412 Myc mutant, either with or without an expression construct encoding full-length Miz-1. Protein stability was determined by pulse–chase analysis as described in Materials and methods. Phosphoimaging was used to determine the percentage of radioactivity present for each Myc–Miz combination at each time point.

the N-terminus of the protein, with maximal degron function residing within the first 128 residues of Myc.

Myc is stabilized by sequences required for association with Miz-1

Our studies showed that C-terminal residues 294–439 of Myc confer protein stability. This region of Myc contains sequences required for Max association and DNA binding (Blackwood and Eisenman, 1991), as well as sequences required for association with the POZ domain protein Miz-1 (Peukert *et al*., 1997). To determine which, if either, of these activities stabilizes Myc, we examined the stability of two finer Myc deletion mutants: Δ412–434, which is defective for association with Max but not Miz-1 (Peukert *et al*., 1997), and Δ370–412, which is defective for association with both Miz-1 and Max (Peukert *et al*., 1997). The results of this analysis are shown in Figure 6A. Deletion of Myc residues 412–434 had little effect on the stability of Myc (compare Δ412–434 with full-length Myc). In contrast, deletion of residues 370–412 markedly reduced Myc stability, decreasing the half-life of the protein to ~15 min (compare Δ370–412 with full-length Myc). The different behavior of these mutants suggests that loss of association with Miz-1, but not with Max, destabilizes Myc.

We next examined whether overexpression of Miz-1 stabilizes Myc. We transiently co-expressed Myc and Miz-1 in HeLa cells, and measured Myc stability by pulse–chase analysis, as summarized in Figure 6B. In the presence of ectopically expressed Miz-1, Myc was indeed significantly more stable. This stabilization required sequences in Myc that associate with Miz-1, because the Δ370–412 Myc mutant, which does not interact with Miz-1 (Peukert *et al*., 1997), was not stabilized by co-expression of Miz-1. Thus, taken together, the data in Figure 6 suggest that Miz-1 acts to stabilize Myc *in vivo*.

Myc is destroyed by ubiquitin-mediated proteolysis in yeast

We next examined whether Myc is unstable in yeast. We reasoned that, as the Myc TAD activates transcription in the yeast *Saccharomyces cerevisiae* (Amati *et al*., 1992), it may also function as a degron in this species. We expressed either full-length Myc or the ΔN2 Myc deletion mutant in the wild-type yeast strain W303-1A, and measured their stabilities using pulse–chase analysis, as shown in Figure 7A. Full-length Myc disappears quickly under these conditions, with an estimated half-life of ~2.5 min, demonstrating that Myc is unstable in yeast. A similar conclusion was reached recently by Flinn *et al*. (1998). As in mammalian cells, Myc turnover in yeast requires the Myc TAD, because deletion of the first 147 residues of Myc stabilized Myc considerably (ΔN2), increasing its half-life to ~20 min. The same degree of stabilization was observed in a yeast strain that carries temperature-sensitive mutations in two β-type (*pre1* and *pre4*) subunits of the proteasome (Hilt *et al*., 1993), as shown in Figure 7B. At the restrictive temperature, Myc was considerably more stable in the proteasome mutant strain (YY374) than in the wild-type congenic control strain (YY371), demonstrating that rapid turnover of Myc in yeast requires a functioning proteasome.

Finally, we asked whether Myc becomes ubiquitylated in yeast. To do this, we used a variant of His-Ub (Willems *et al*., 1996), in a manner analogous to the human cell assay presented in Figure 2. The results of this analysis are shown in Figure 7C. Full-length Myc was clearly ubiquitylated in this assay, as revealed by the smear of high molecular weight, HA-tagged material in the presence of His-Ub (lane 10), but not its absence (lane 9). As in human cells, deleting the first 147 residues of Myc (ΔN2) blocked high levels of ubiquitylation in yeast (compare lanes 10 and 12). We therefore conclude that, as in human cells, the Myc TAD is required for Ub-mediated Myc destruction in yeast.

Cancer-associated and transforming mutations within the Myc degron stabilize Myc

Mutations within exons 2 and 3 of c-*myc* occur in a large number of Burkitt's and other lymphomas (Bhatia *et al*., 1993; Clark *et al*., 1994). The majority of these mutations alter sequences within the first 130 amino acid residues of Myc. Our demonstration that this region of Myc signals its destruction by Ub-mediated proteolysis raised the

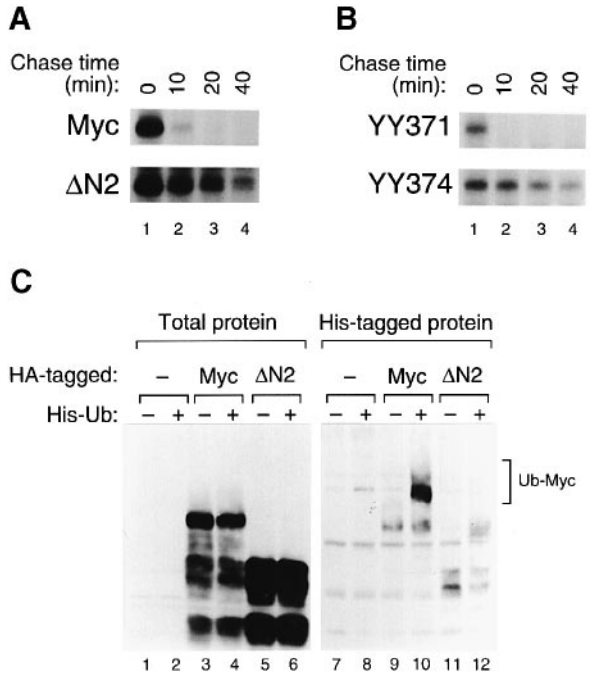

Fig. 7. The transcriptional activation domain of Myc signals Ub-mediated proteolysis in yeast. (**A**) Myc is unstable in yeast. Wild-type yeast strain W303-1A were transformed with expression constructs encoding either HA epitope-tagged full-length Myc or the ΔN2 Myc deletion mutant, and protein stabilities determined by pulse–chase analysis, as described in Materials and methods. (**B**) Myc is stabilized by genetic disruption of the proteasome. Wild-type yeast strain YY371 and *pre1-1*, *pre4-1* double mutant yeast strain YY374, each expressing epitope-tagged Myc, were placed at the restrictive temperature of 37°C. Thirty minutes after placing cells at 37°C, Myc stability was determined by pulse–chase analysis. (**C**) The transcriptional activation domain of Myc is required for Myc ubiquitylation in yeast. Wild-type (W303-1A) yeast expressing HA-tagged full-length (lanes 3, 4, 9 and 10) and ΔN2 Myc (lanes 5, 6, 11 and 12) were transformed with an expression construct encoding untagged ubiquitin ('–'; odd-numbered lanes) or His-tagged ubiquitin ('+'; even-numbered lanes). Cell lysates were prepared, and total (lanes 1–6) and histidine-tagged (lanes 7–12) proteins prepared as described in Materials and methods and in the legend to Figure 2. Proteins were resolved by SDS–PAGE and HA-tagged proteins detected by immunoblotting.

possibility that these mutations disrupt Myc degron function, and thus stabilize Myc. To determine if cancer-associated and transforming Myc mutations can alter its stability, we examined the stability of several different Myc alleles: (i) T58A, which is found in several viral Myc proteins and a Burkitt's lymphoma cell line (Pulverer *et al*., 1994); (ii) A21, a complex mutation (S71Y, L82H, Δ115–120) identified in a tumor sample from a patient suffering from an aggressive, AIDS-associated lymphoma (Clark *et al*., 1994); (iii) A44V, a mutation discovered in a Burkitt's lymphoma patient and in a separately derived Burkitt's lymphoma cell line (Bhatia *et al*., 1993); (iv) P57S, a mutation discovered in a Burkitt's lymphoma patient (Bhatia *et al*., 1993); and (v) D4 (mutations N127D and I129L), identified in a patient suffering from an aggressive, non-AIDS-associated lymphoma (Clark *et al*., 1994).

We expressed the mutant Myc alleles in U2OS cells and measured their stability as shown in Figure 8. With the exception of the D4 allele, all mutant Myc proteins were more stable that their wild-type counterpart. The P57S mutation had the smallest effect on Myc stability,

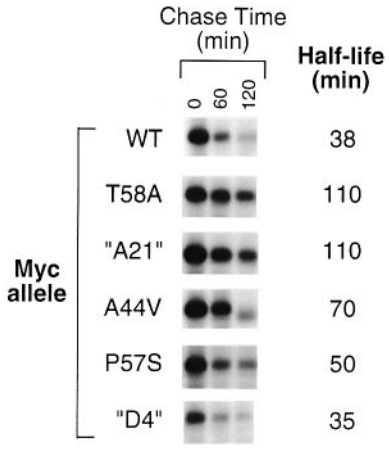

Fig. 8. Cancer-associated and transforming Myc mutations increase Myc stability. Human U2OS cells were transiently transfected with expression constructs encoding wild-type human Myc (WT) or the indicated mutant Myc alleles. Protein stability was determined by pulse–chase analysis as described in Materials and methods.

increasing the half-life of Myc in these cells from 38 to 50 min. The largest effects on Myc stability were observed with the T58A and A21 mutations, which increased the half-life of Myc to 110 min each. Thus, cancer-associated and transforming mutations within Myc stabilize the protein *in vivo*. Moreover, the finding that several different mutations stabilize Myc suggests that protein stabilization is a common consequence of cancer-associated mutations within the Myc degron.

Discussion

Myc is a potent regulator of cellular proliferation. Accordingly, cells have evolved numerous mechanisms to keep the activity of Myc tightly in check. The destruction of Myc by Ub-mediated proteolysis is one of these mechanisms. Here, we have shown that Myc stability is regulated by the action of two opposing elements: a transcriptional activation domain that signals Myc destruction, and a region required for association with Miz-1 that stabilizes Myc. We have also found that several cancer-associated and transforming mutations within the activation domain of Myc stabilize the protein. Our results expose a complex pattern of interactions that regulate Myc stability and reveal an unexpected mechanism that could contribute to oncogenic transformation by Myc.

The Myc degron

While this manuscript was in preparation, Flinn *et al*. (1998) reported that two conserved regions within Myc, referred to as Myc boxes I (MbI; residues 45–63) and II (MbII; residues 126–144), are degrons in yeast and mammalian cells, and concluded that these boxes are signals for the rapid turnover of Myc. Our data are not entirely consistent with this model. For example, although MbII can signal protein destruction (Figure 5), it does not appear to be dominant in directing the destruction of full-length Myc. We have mapped the Myc degron to a region within the first 128 residues of Myc, which excludes MbII, and have shown that deletion of MbII from Myc does not increase Myc stability *in vivo* (unpublished observations). We have also found that, when fused to the GAL4 DBD,

MbII decreases the activity of the Myc degron (Figure 5, compare Myc residues 2–128 with 2–147), arguing that MbII does not play a positive role in Myc degron function *in vivo*. Finally, we note that the tumor-associated Myc allele A21 has no mutations within either MbI or MbII, but is significantly more stable that wild-type Myc (Figure 9). These findings reveal that the Myc degron does not require MbII for function, and appears to be more extensive than just MbI itself. We have been unable, in these experiments and others (unpublished observations), to define a 'core' region uniquely required for Myc degron function, and conclude that the Myc degron, like its TAD, is composed of multiple elements that function in concert to promote Myc destruction.

The location of the Myc degron is intriguing, because residues 1–128 of Myc serve to integrate many of the signals that regulate Myc function, such as binding to the retinoblastoma-related repressor protein p107 (Beijersbergen *et al*., 1994), and cell cycle- and growth-regulated phosphorylation (Seth *et al*., 1991; Henriksson *et al*., 1993; Pulverer *et al*., 1994; Alexandrov *et al*., 1997). It is thus conceivable that, in addition to regulating other aspects of Myc activity, these regulatory mechanisms also modulate Myc stability. Indeed, given that Rb-related proteins regulate the transcriptional activity and stability of E2F-1 and -4 (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Campanero and Flemington, 1997), and considering the frequent involvement of phosphorylation in Ub-mediated proteolysis (Hochstrasser, 1996; Elledge and Harper, 1998), our findings suggest that Myc stability is regulated, either positively or negatively, by growth-related signaling to its degron.

The location of the Myc degron also raises the interesting question of the nature of its overlap with the Myc TAD. We suspect that degron and TAD function are linked because of the correlation we observe between the ability of individual regions of the Myc TAD/degron to activate transcription and to signal protein destruction (Figure 5). We also note that we are not the first to observe an overlapping TAD and degron: in their analysis of E2F-1 destruction, Campanero and Flemington (1997) mapped the E2F-1 degron to sequences overlapping the E2F-1 TAD, and proposed that this overlap might reflect the involvement of the Ub–proteasome pathway in transcription. Perhaps the TAD-directed destruction of Myc and E2F-1 reflects a conserved mechanism in which transcription factors function, in part, by recruiting factors that ubiquitylate histones, basal factors and themselves.

Stabilization of Myc by Miz-1

Residues within the C-terminus of Myc promote protein stability (Figures 3 and 4). We have mapped this activity to within a region of Myc required for association with Miz-1 (Figure 6) and have shown that overexpression of Miz-1 stabilizes Myc. Together, these data suggest that the association of Myc with Miz-1 stabilizes Myc.

What is the significance of Myc stabilization by Miz-1? Miz-1 is a zinc finger/POZ domain protein (Peukert *et al*., 1997) that, alone, binds to and transactivates the cyclin D1 promoter. When Miz-1 is complexed with Myc, however, a latent Miz-1 repressor function is revealed, and cyclin D1 promoter activity is attenuated. Because of this behavior, Miz-1 has been proposed as the partner protein through which Myc represses transcription of cyclin D1 (Philipp *et al*., 1994) and other genes *in vivo*. We hypothesize that stabilization of Myc by Miz-1 relates to the function of Miz-1–Myc complexes as transcriptional repressors. An analogous situation is observed with E2F (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Campanero and Flemington, 1997), which is an unstable transcriptional activator in the absence of Rb, but a stable transcriptional repressor in its presence. Perhaps, therefore, enhanced protein stability is a common feature of transcriptional repressors, permitting the formation of recalcitrant complexes that stably attenuate gene transcription. Undoubtedly, the significance of Myc stabilization by Miz-1 will become clearer once more is known about Miz-1 itself.

Myc degron function in cancer

Our observation that the Myc degron overlaps a region of Myc frequently mutated in human cancers prompted us to examine how cancer-associated mutations affect Myc stability. Out of five mutant Myc alleles tested, four were more stable that wild-type Myc, suggesting that reduced Myc turnover is a common consequence of cancer-associated changes in the Myc degron.

Not all mutations affect Myc destruction to similar extents. The A44V and P57S mutations increase Myc half-life to ~1 h. The T58A and A21 mutations increase Myc half-life to almost 2 h (Figure 8). In all cases, these increases were concomitant with an increase in the steady-state levels of Myc protein (unpublished observations). The extent of stabilization by the T58 and A21 mutations is comparable to that observed with wild-type Myc in the presence of proteasome inhibitor (Figure 2), or when the entire Myc degron is deleted (Figure 4), arguing that these mutations substantially disable Myc degron function.

The A21 allele is quite complex, carrying two point mutations (S71Y and L82H) and a deletion of residues 115–120 (Bhatia *et al*., 1993) that together encompass a large portion of the Myc degron. We have found that all three of these mutations are required to stabilize Myc (unpublished observations), suggesting that it is the combined effect of disrupting individual elements within the Myc degron that stabilizes the A21 allele. In contrast, however, the T58A allele carries only a single amino acid substitution at residue T58, yet it is as stable as the A21 allele. If individual elements within the Myc degron can signal protein destruction, why then should Myc turnover be so sensitive to mutation at one residue?

We suggest that residue T58 functions to coordinate the activity of the entire Myc degron. Residue T58, together with nearby residue S62 (Lutterbach and Hann, 1994), are major sites of Myc phosphorylation *in vitro* and *in vivo* (Lutterbach and Hann, 1994; Pulverer *et al*., 1994). Many kinases have been shown to phosphorylate T58/S62 *in vitro*, including mitogen-activated protein (MAP) kinases (Lutterbach and Hann, 1994), the $p34^{cdc2}$ kinase (Lutterbach and Hann, 1994), glycogen synthase kinase 3 (GSK-3; Henriksson *et al*., 1993; Pulverer *et al*., 1994) and a p107–cyclin A–CDK complex (Hoang *et al*., 1995). Because substrate phosphorylation frequently is required for protein destruction by Ub-mediated proteolysis (Hochstrasser, 1996; Elledge and Harper, 1998), we suggest that phosphorylation of residue 58 of Myc is required

for its rapid destruction. This possibility is currently being explored.

Regardless of the mechanism, the increased stability of cancer-associated and transforming Myc alleles suggests that disruption of Myc destruction is a mechanism through which Myc can become activated. Although we do not know whether these forms of Myc are more stable in cancer cells, our finding that the T58A mutation stabilizes Myc considerably (Figure 8) strongly implies a link between enhanced Myc stability and oncogenic transformation by Myc. The T58A mutation has been shown to increase the transforming ability of c-Myc considerably (Pulverer *et al*., 1994). Moreover, this mutation is naturally found in several viral forms of Myc, and its replacement with threonine in these v-Myc proteins substantially reduces their oncogenic potential (Palmieri *et al*., 1983). It has been suggested that the T58A mutation is transforming because it disrupts transcriptional repression by p107 (Hoang *et al*., 1995), although these results have been disputed (Smith-Sorensen *et al*., 1996). Our data suggest a new and simple way in which the T58A mutation increases Myc's transforming activity: by blocking Myc destruction, leading to increased Myc abundance, and deregulated expression of Myc target genes. A similar scenario could also apply to the other stable alleles we have identified. Given the frequent occurrence of mutations within the Myc degron in cancer, we suggest that enhanced protein stability is a common mechanism contributing to oncogenic transformation by Myc.

Materials and methods

Plasmid DNA manipulations

To create a mammalian expression vector encoding HA-epitope-tagged Myc, sequences encoding residues 2–439 of human c-*myc* were PCR-amplified from the plasmid pUHD10.3 huMyc (provided by R.Maestro, Centro di Riferimento Oncologico, Aviano, Italy) and subcloned into the mammalian expression vector pCGN (Tanaka and Herr, 1990), creating the vector pCGN-Myc. All Myc deletion and point mutants were generated by site-directed mutagenesis (Kunkel *et al*., 1987) of this pCGN-Myc construct. To construct mammalian expression vectors encoding epitope-tagged GAL4–Myc derivatives, the vector pCG-GAL(1–94) (Das *et al*., 1995) was first modified by addition of sequences encoding the HA epitope tag at the C-terminus of the GAL4 DBD; appropriate Myc-coding sequences were then subcloned into this vector. To create yeast Myc expression vectors, HA-Myc encoding sequences were excised from the appropriate pCGN-Myc construct and subcloned into the *Spe*I and *Bam*HI sites of p426GPD (Mumberg *et al*., 1995; kindly supplied by M.Funk, Institut für Molekularbiologie und Tumorforschung, Marburg, Germany). The resulting plasmids expressed HA-tagged Myc under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter.

Antibodies

To generate polyclonal anti-Myc antibodies, a synthetic peptide corresponding to human Myc sequences 424–439 (sequence: REQLKHKLEQLRNSCA) was coupled to keyhole limpet hemocyanin (Pierce) and injected into rabbits. The resulting antiserum (#680) was characterized by immunoblotting. Mouse monoclonal anti-HA antibody 12CA5 was provided by C.Bautista (CSHL). Rabbit polyclonal anti-TFIIB antisera was provided by T.Kuhlman and N.Hernandez (CSHL). Rabbit polyclonal anti-p53 antibody (FL-393) was purchased from Santa Cruz.

Analysis of endogenous Myc protein levels

Mouse *H38-5* and *ts20TG*^{R} cells, provided by H.Ozer (University of Medicine and Dentistry, NJ), were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-10 medium at the permissive temperature of 35°C as described previously (Chowdary *et al*., 1994). For each experiment, *H38-5* and *ts20TG*^{R} cells were seeded at a density of 1×10^{6} cells/10 cm dish, and grown for 24 h at 35°C. Cells were then re-fed with fresh media and transferred to the restrictive temperature of 39.5°C. At the indicated time points, cells from one dish each of *H38-5* and *ts20TG*^{R} cells were harvested and lysed directly in SDS–PAGE loading buffer. Equivalent amounts of cell lysate were resolved by SDS–PAGE, and Myc, p53 and TFIIB levels quantitated by immunoblotting.

Human HeLa and U2OS cells were grown in DMEM supplemented with 10% fetal calf serum (FCS). A total of 1×10^{6} cells/10 cm dish were treated with protease inhibitors LLnL (Calbiochem), LLM (Calbiochem), MG132 (Calbiochem), E64 (Sigma) or proteasome inhibitor I (PSI; Calbiochem), each dissolved in dimethylsulfoxide (DMSO). Inhibitors were applied for 12 h at a final concentration of 25 μM each. Following treatment, cells were harvested, and steady-state Myc and TFIIB levels quantitated as described above.

Determination of protein stability

For each protein to be assayed, four 6 cm dishes of cells, each containing ~5×10^{5} cells, were transfected (Tanaka *et al*., 1992) with (i) 80 ng of the appropriate pCGN-Myc or pCG-GAL(1–94)HA-Myc derivative, (ii) 800 ng of the Miz-1 expression construct pcDNA3.Miz-1 (kindly provided by M.Eilers, Institut für Molekularbiologie und Tumorforschung, Universität Marburg, Germany) (Peukert *et al*., 1997) where indicated, and (iii) 10 μg of pUC119. Pulse–chase analysis was begun 34 h post-transfection. Where indicated, the proteasome inhibitor MG132 was applied to cells at a final concentration of 50 μM for 2 h prior to the pulse–chase. To perform the pulse–chase, cell monolayers were washed twice with warm phosphate-buffered saline (PBS), and starved of methionine and cysteine by incubation for 40 min at 37°C in 1 ml of methionine/cysteine-free DMEM (Gibco-BRL), supplemented with 5% dialyzed FCS (Gibco-BRL). Following amino acid starvation, cellular proteins were pulse-labeled by incubating each plate of cells with 400 μCi of [^{35}S]methionine/cysteine (Tran^{35}S-label; NEN) for 30 min at 37°C. The radioactive medium was then removed, the cells washed twice in warm PBS, re-fed with DMEM supplemented with 2 mM methionine and 2 mM cysteine, and incubated for the indicated times. Cells were collected, and labeled proteins recovered by denaturing immunprecipitation using the 12CA5 antibody and the method of Hofmann *et al*. (1996). Immune complexes were detected by SDS–PAGE and autoradiography.

The stabilities of full-length Myc and the ΔN2 Myc deletion mutant in yeast were determined using pulse–chase analysis. The appropriate p426GPD expression constructs were transformed into the yeast strain W303-1A (*MAT***a***; ura3-1; leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100*) and the transformants maintained on selective media. These constructs were also transformed into the double proteasome mutant strain YY374 (*MAT***a***; ura3-1; leu2-3,-112, his3-11,-15; pre1-1; pre4-1*) and its congenic control YY371 (*MAT***a***; ura3-1; leu2-3,-112, his3-11,-15; pre1-1; pre4-1*) (Yaglom *et al*., 1995), kindly provided by D.Finley (Harvard Medical School, MA). To perform the pulse–chase, 20 ml cultures for each transformant were grown at the appropriate temperature to mid-log phase. Cells were collected by centrifugation, resuspended in selective media without methionine, and grown at the appropriate temperature for 1 h. Cells were then collected and resuspended in selective media without methionine to give a final OD_{600} of 6.0. A 1 ml aliquot of each cell suspension was incubated with 1 mCi of [^{35}S]methionine/cysteine (Tran^{35}S-label) for 10 min at the appropriate temperature. Cells were then collected, transferred to 6 ml of media containing an additional 1 mg/ml of unlabeled methionine, and incubation continued. At the indicated time points, 1.4 ml aliquots of cells were collected by centrifugation, and labeled proteins recovered by denaturing immunoprecipitation using the 12CA5 antibody and the method of Chen *et al*. (1993). For the YY371 and YY374 strains, cultures were shifted to 37°C for 30 min prior to performing the pulse–chase.

Normalizing steady-state RNA and protein levels

To determine the steady-state level of GAL4–Myc fusion proteins as a function of steady-state RNA levels, transfected HeLa cells were harvested and divided into two aliquots. One sample was used to prepare total proteins. The other sample was used to prepare cytoplasmic RNA as described (Tanaka *et al*., 1992). Northern blotting was used to determine the relative levels of RNA for each GAL4–Myc derivative. We then normalized the RNA samples to give equivalent signal strengths and confirmed the success of normalization by performing a second Northern blot. The normalization value applied to each RNA sample was then used to adjust the amount of total proteins analyzed by SDS–PAGE and immunoblotting.

Analysis of transcriptional activation

To determine the transcriptional activity of GAL4–Myc derivatives, ~1×10^6 HeLa cells (in a 10 cm dish) were transfected with: (i) 1 μg of the reporter plasmid p4xGal.c-*fos*.TAT.luc (gift of A.Berk, University of California, Los Angeles, CA; Bryant *et al*., 1996), (ii) pSVβgal (gift of G.Hannon, CSHL), (iii) 500 ng of the appropriate pCG-GAL(1–94)HA-Myc expression plasmid, and (iv) pUC119, taking the total amount of DNA to 20 μg. At 40 h after transfection, cells were harvested and luciferase and β-galactosidase activities determined using luciferase and β-galactosidase assay systems (Promega). The relative transcriptional activities for each activator were calculated by dividing the luciferase activity for each sample by the β-galactosidase activity.

Detection of ubiquitin conjugates

To detect ubiquitylated proteins in human cells, we used the His_6-tagged Ub method of Treier *et al*. (1994), as modified by Campanero and Flemington (1997). Briefly, 1×10^6 HeLa cells were transfected with 2 μg of each pCGN-Myc or pCG-GAL(1–94)HA-Myc expression construct, in either the presence or absence of 4 μg of the plasmid pMT107 (gift of D.Bohmann, EMBL, Heidelberg, Germany), which encodes His-tagged human Ub. At 40 h after transfection, cells were harvested and total histidine-tagged proteins purified on nickel-NTA–agarose (Qiagen) exactly as described (Campanero and Flemington, 1997). HA-tagged proteins were detected by SDS–PAGE and immunoblotting using the 12CA5 anti-HA antibody. The plasmid pMT108 (gift of D.Bohmann), which encodes HA-tagged c-Jun, was a positive control for these assays.

To detect ubiquitylated proteins in yeast, W303-1A transformants carrying p426GPD expression vectors were transformed with plasmids pUB204 or pUB223 (kindly provided by D.Finley). Both vectors express K48R/G76A mutant Ub under the control of the copper-inducible *CUP1* promoter: pUB204 expresses untagged Ub, and pUB223 expresses Ub carrying the polyhistidine tag (Willems *et al*., 1996). Strains were grown to early-log phase in selective media, and the *CUP1* promoter induced by the addition of 250 μM copper sulfate. After 5 h of induction, cultures were harvested. Total proteins were extracted from 5 ml of culture by glass bead lysis in SDS–PAGE loading buffer. Proteins from the remaining culture were solubilized in buffer A (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole), and total His-tagged proteins purified on nickel-NTA–agarose exactly as described (Willems *et al*., 1996). Total proteins and His-tagged proteins were then resolved by SDS–PAGE and detected by immunoblotting using the 12CA5 anti-HA antibody.

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