# A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells

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The stability of c-Myc is regulated by multiple Ras effector pathways. Phosphorylation at Ser 62 stabilizes c-Myc, whereas subsequent phosphorylation at Thr 58 is required for its degradation. Here we show that Ser 62 is dephosphorylated by protein phosphatase 2A (PP2A) before ubiquitination of c-Myc, and that PP2A activity is regulated by the Pin1 prolyl isomerase. Furthermore, the absence of Pin1 or inhibition of PP2A stabilizes c-Myc. A stable c-Myc$^{T58A}$ mutant that cannot bind Pin1 or be dephosphorylated by PP2A replaces SV40 small T antigen in human cell transformation and tumorigenesis assays. Therefore, small T antigen, which inactivates PP2A, exerts its oncogenic potential by preventing dephosphorylation of c-Myc, resulting in c-Myc stabilization. Thus, Ras-dependent signalling cascades ensure transient and self-limiting accumulation of c-Myc, disruption of which contributes to human cell oncogenesis.

Temporal regulation of c-Myc protein accumulation is essential for normal cell proliferation. Complete loss of c-Myc or N-Myc function results in embryonic lethality[1,2], and studies using conditional c-Myc knockout alleles show that cells lacking c-Myc cease to proliferate and exit the cell cycle[3,4]. In contrast, overexpression of c-Myc in cultured cells or transgenic animals blocks differentiation, induces neoplastic transformation and can initiate apoptosis[5,6]. Moreover, a wide variety of naturally occurring tumours exhibit both chromosomal translocations and amplification of the c-*myc* locus that result in constitutive overexpression of c-Myc protein[7–9].

Myc protein is stabilized after activation of Ras, allowing it to accumulate to high levels[10]. Ras promotes stability of c-Myc through at least two effector pathways: the Raf–MEK–ERK kinase cascade, and the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway that inhibits glycogen synthase kinase-3β (GSK-3β)[11]. The ERK and GSK-3β kinases phosphorylate two sites near the amino terminus of c-Myc that are highly conserved in all mammalian c-Myc isoforms[11–14]. These phosphorylation sites, Thr 58 and Ser 62, exert opposing control on c-Myc degradation through the ubiquitin-proteasome pathway[11]. Thus, after a growth stimulatory signal, c-*myc* gene transcription is increased and newly synthesized c-Myc protein is phosphorylated on Ser 62, resulting in its stabilization. Phosphorylation at Ser 62 is also required

for the subsequent phosphorylation of c-Myc at Thr 58 by GSK-3β, which is associated with c-Myc degradation[11–14]. During early G1 phase, however, GSK-3β activity is regulated by Ras-mediated activation of the PI(3)K/Akt pathway (which phosphorylates and inhibits GSK-3β), facilitating stabilization of c-Myc[15]. Later in G1 phase, when Akt activity declines, GSK-3β becomes active and phosphorylates c-Myc on Thr 58, which is important for c-Myc turnover.

Thr 58 phosphorylation contributes to degradation of c-Myc through the ubiquitin pathway, as mutation of Thr 58 to alanine results in a stable and more oncogenic c-Myc protein[11–13,16,17]. Moreover, the c-Myc$^{T58A}$ mutant is no longer a substrate for ubiquitination *in vivo*[11]. Nevertheless, how Thr 58 phosphorylation facilitates c-Myc degradation remains unclear. We now show that Ser 62 dephosphorylation is important for ubiquitin-mediated degradation of c-Myc and that Thr 58 phosphorylation promotes dephosphorylation of Ser 62. In addition, we show that PP2A dephosphorylates Ser 62 on c-Myc and that the Pin1 prolyl isomerase, which has previously been shown to enhance PP2A function[18,19], also regulates c-Myc turnover in a process that is dependent on phosphorylation of Thr 58. We propose that a sequence of Ras-initiated signalling events is required to regulate c-Myc protein stability and demonstrate that a stabilized c-Myc protein can replace SV40 small T antigen in the transformation of human cells.

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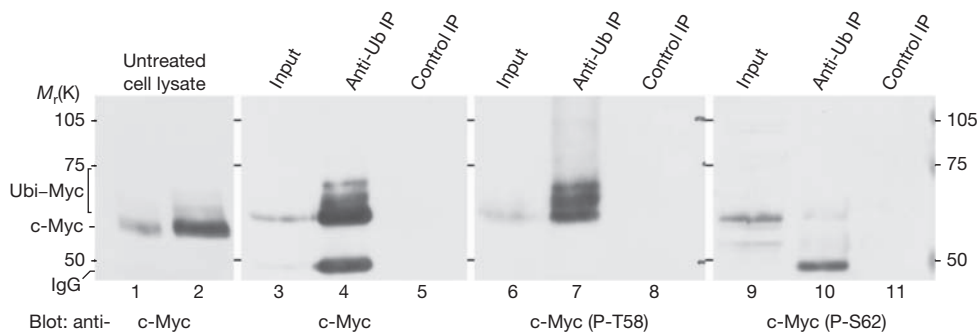[7]Correspondence should be addressed to R.S. (e-mail: searsr@ohsu.edu).

**Figure 1** Ubiquitinated c-Myc protein is phosphorylated on Thr 58, but not on Ser 62. Quiescent REF 52 fibroblasts were infected with Ad-c-Myc (MOI = 50). At 16 h after infection, cells maintained in low-serum medium were treated with 10 μM lactacystin for 6 h, except for the samples shown in lanes 1 and 2, which represent 10 and 40 μg of untreated Ad-c-Myc-infected cell lysate, respectively. Lactacystin-treated cells were harvested and lysates were divided for immunoprecipitation (IP) with an anti-ubiquitin antibody or control protein A + G beads. The input lysates (10% volume), anti-ubiquitin IP, and control IP were then analysed in triplicate by SDS–PAGE and western blotting with an anti-c-Myc monoclonal antibody (C-33/sc-42), or one of the two phospho-specific c-Myc antibodies[11]. The c-Myc and ubiquitinated c-Myc proteins are indicated. The 50K band visible in lanes 4 and 10 is the IgG heavy chain, which is recognized by the anti-mouse secondary antibody. This band is not visible in lane 7 and only faintly visible in lane 10 (longer exposure), where anti-rabbit secondary antibodies were used.

## RESULTS

### Ubiquitinated c-Myc is phosphorylated on Thr 58, but not on Ser 62

Newly synthesized c-Myc is phosphorylated on Ser 62, allowing the subsequent phosphorylation of Thr 58 that is necessary to promote ubiquitin-mediated degradation of c-Myc[11–14]. However, treatment of cells with a proteasome inhibitor results in the accumulation of species of c-Myc with a larger relative molecular mass higher ($M_r$) that is recognized by an antibody specific for Thr 58-phosphorylated c-Myc, but not by an antibody specific for Ser 62-phosphorylated c-Myc[11]. This observation suggested that ubiquitinated forms of c-Myc might be phosphorylated selectively on Thr 58. To address this possibility, we examined the phosphorylation status of ubiquitinated c-Myc.

A recombinant adenovirus containing the *c-myc* gene under the control of the cytomegalovirus (CMV) enhancer (Ad-Myc) was used to produce c-Myc protein in otherwise quiescent cells, an approach we have employed previously to control the conditions in which c-Myc accumulates[10,11]. We have shown that c-Myc is phosphorylated at Ser 62 as well as Thr 58 under these conditions, and that serum stimulation or mutation of Thr 58 increases Ser 62 phosphorylation[11]. Here, Ad-Myc-infected cells were treated with the proteasome inhibitor lactacystin for 6 h and ubiquitinated proteins were immunoprecipitated from cell lysates with an anti-ubiquitin antibody. The immunoprecipitates, along with 10% of the original lysate and control immunoprecipitates without the ubiquitin antibody, were analysed in triplicate by SDS–PAGE and immunoblotting with either a pan-Myc antibody, a phospho-Thr 58 antibody or a phospho-Ser 62 antibody. A substantial amount of ubiquitinated c-Myc accumulates after 6 h of proteasome inhibition (Fig. 1, lane 4). Ubiquitinated c-Myc runs with a slightly higher relative molecular mass than c-Myc produced in the absence of proteasome inhibition (Fig. 1, compare lanes 2 and 4). In addition, several higher-molecular-weight species are detected with the pan-Myc antibody that we assume represent alternate or additional ubiquitinated species of c-Myc. Ubiquitinated c-Myc, which accumulates in quiescent cells after proteasome inhibition, is primarily (if not exclusively) phosphorylated on Thr 58 (Fig. 1, lane 7), as there was little or no Ser 62-phosphorylated c-Myc immunoprecipitated by the ubiquitin antibody (Fig. 1, lane 10).

### A role for PP2A in controlling c-Myc degradation

The observations that GSK-3β–mediated phosphorylation at Thr 58 requires earlier phosphorylation of c-Myc at Ser 62 (refs 11–14) and that ubiquitinated c-Myc is only phosphorylated at Thr 58 suggest that the Ser 62 phosphate is removed in the process of c-Myc ubiquitination. Assays using various cell-permeable inhibitors specific for common serine/threonine phosphatases indicated a possible role for PP2A in the control of c-Myc stability. The level of c-Myc present in quiescent cells was increased by okadaic acid (Fig. 2a, compare lanes 1 and 2), which is a potent inhibitor of PP2A with some activity against PP1, but was not affected by cyclosporin A (Fig. 2a, lane 3), which specifically inhibits PP2B. This data suggest that PP2A, or possibly PP1, could be involved in controlling c-Myc protein accumulation.

As a more specific test to examine the role of PP2A in controlling accumulation of c-Myc, we used a recombinant adenovirus that expresses SV40 small T antigen, a highly selective inhibitor of PP2A. Small T antigen functions by displacing specific regulatory B subunits that normally incorporate into cellular PP2A complexes and thereby reduces PP2A activity against certain endogenous substrates[20]. Increasing doses of the Ad-small T antigen virus had a marked effect on c-Myc protein levels, resulting in a substantial increase in the accumulation of c-Myc in quiescent fibroblasts (Fig. 2b). The effects of expressing small T antigen on accumulation of endogenous c-Myc in quiescent or asynchronous REF52 fibroblasts were also examined. Endogenous c-Myc accumulation was increased substantially by inhibition of PP2A with small T antigen in either serum-starved or asynchronously growing cells (Fig. 2c).

To test whether the increase in c-Myc accumulation observed after PP2A inhibition is caused by protein stabilization, and to rule out effects of viral infection on the interpretation of our results, the effect of okadaic acid on the half-life of endogenous c-Myc protein was examined by pulse-chase analysis in quiescent REF52 fibroblasts. c-Myc protein produced in growth-arrested cells was short-lived, with a half-life of 13 min. Importantly, this was true for endogenous c-Myc in uninfected cells (Fig. 2d, top), as well as for c-Myc produced by adenovirus (Fig. 2d, bottom). In contrast, endogenous c-Myc produced in uninfected quiescent cells treated with okadaic acid was significantly more stable, with a half-life of 62 min. A similar stabilization of c-Myc was observed in cells infected with the Ad-small T antigen virus (see
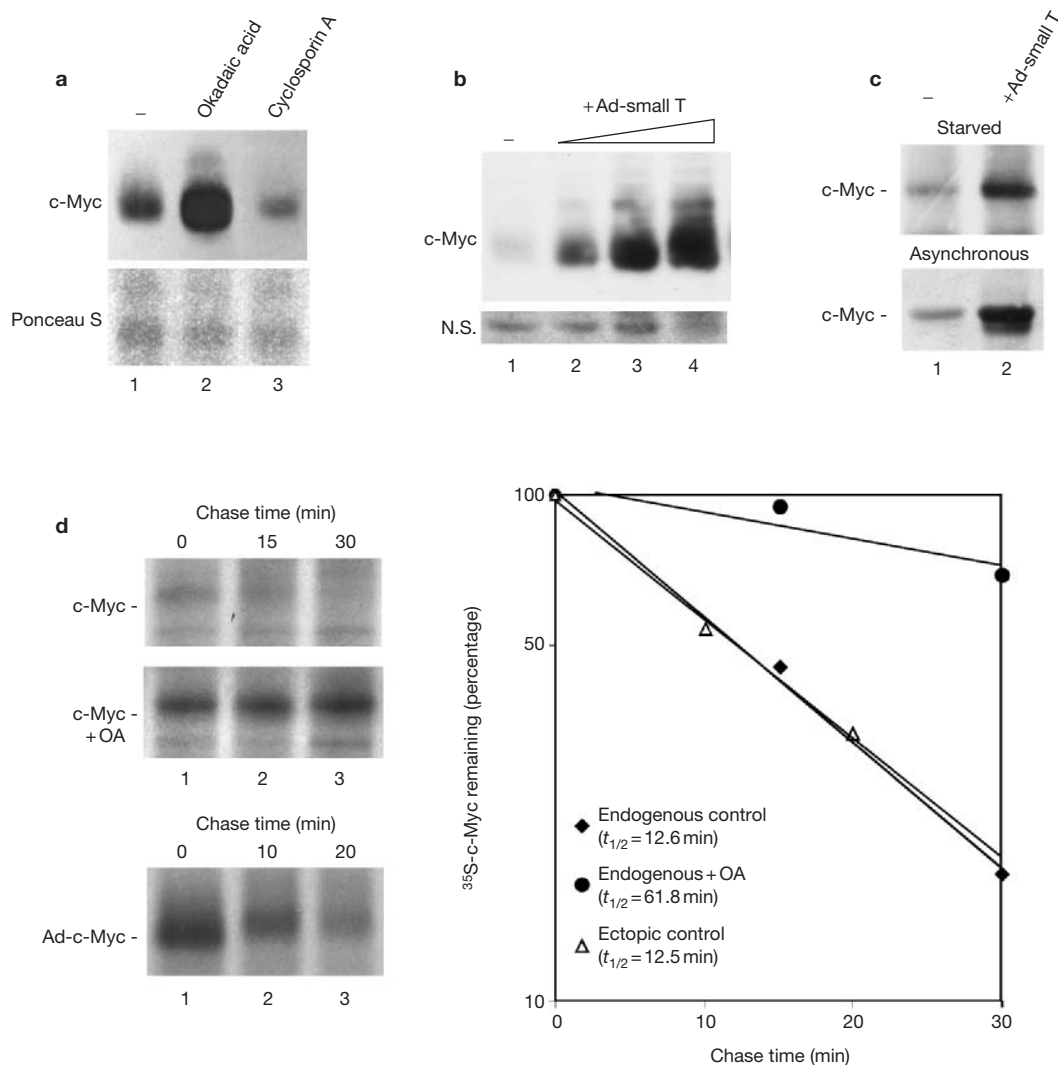
**Figure 2** Accumulation of c-Myc is regulated by PP2A. (**a**) Okadaic acid (OA) increases accumulation of c-Myc. Quiescent REF52 fibroblasts were infected with Ad-c-Myc (MOI = 25) and maintained in medium with 0.25% serum for an additional 20 h. At 4 h before harvesting, infected cells were either treated with ethanol (–), 100 nM OA or 5 μM cyclosporin A. Extracts from an equal number of cells were subjected to western blot analysis with the c-Myc C-33 antibody. Equal protein loading was verified by Ponceau S staining. (**b**) SV40 small T antigen increases Ad-c-Myc protein levels. Quiescent REF52 cells were infected with Ad-c-Myc (MOI = 25), together with a control virus (–), Ad-GFP (MOI = 200), or a recombinant adenovirus expressing SV40 small T antigen, Ad-small T (MOIs = 100, 200 and 400 in lanes 2–4), and maintained in medium containing 0.25% serum. At 20 h after infection, equal cell counts were harvested for western blot analysis with the C-33 c-Myc antibody. Equal protein loading was verified by Ponceau S staining of the membrane and the presence of a non-specific (N.S.) band on the blot. (**c**) SV40 small T antigen increases endogenous c-Myc protein levels. Quiescent or asynchronous REF52 cells were infected

with either control Ad-GFP virus (–) or Ad small T antigen, both at MOI = 200, and maintained in growth medium (asynchronous) or starvation medium (starved) for an additional 20 h. Equal cell numbers were harvested and immunoprecipitations were performed to concentrate endogenous c-Myc, before western blot analysis with the c-Myc C-33 monoclonal antibody. (**d**) Okadaic acid stabilizes endogenous c-Myc protein. Quiescent REF52 fibroblasts were either infected with Ad-c-Myc (MOI = 50) and maintained in medium with 0.25% serum for 18 h, or not infected. Non-infected cells were either mock treated with ethanol (control) or treated with 100 nM okadaic acid (OA) for 6 h. Cells were pulse-labelled with ^{35}S-methionine/cysteine and chased with medium containing excess unlabelled methionine and cysteine in the continued presence of ethanol or okadaic acid. ^{35}S-labelled c-Myc was immunoprecipitated, separated by SDS–PAGE and quantified with a phosphorimager. Best-fit exponential lines are shown for endogenous c-Myc control, endogenous c-Myc + OA and ectopic c-Myc control. Calculated half-lives are also shown.

Supplementary Information, Fig. S1).

*In vitro* phosphatase assays were then performed to examine the role of PP2A in targeting the Ser 62 phosphate. c-Myc protein was immunoprecipitated from Ad-Myc-infected quiescent REF52 fibroblasts that were co-infected with Ad-Ras to maximize phosphorylation of c-Myc[10]. Immunoprecipitated c-Myc was incubated with either

buffer only (–), or with purified PP1 or PP2A. Ser 62 and Thr 58 phosphorylation were assessed by western blotting with the Ser 62 or Thr 58 phospho-specific antibodies. PP2A, but not PP1, removed Ser 62 phosphate from c-Myc without significantly reducing Thr 58 phosphate (Fig. 3a). The ability of PP2A to dephosphorylate the stable c-Myc mutant, c-Myc[T58A], was also evaluated. This mutant has enhanced Ser
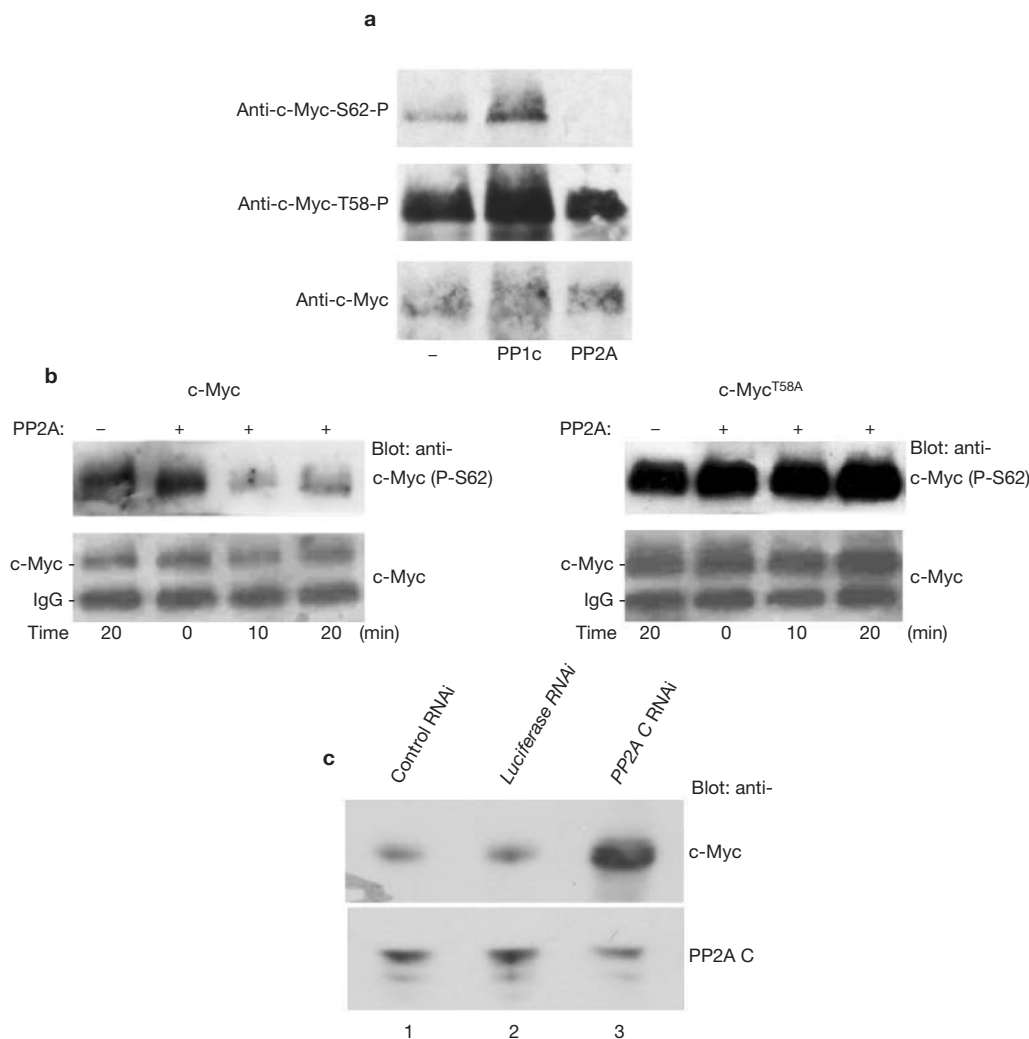
**a**



Anti-c-Myc-S62-P

Anti-c-Myc-T58-P

Anti-c-Myc

    &minus;    PP1c  PP2A

**b**

c-Myc

PP2A:  &minus;    +    +    +

Blot: anti-c-Myc (P-S62)

c-Myc
IgG

c-Myc

Time   20    0   10   20  (min)

c-Myc$^{T58A}$

PP2A:  &minus;    +    +    +

Blot: anti-c-Myc (P-S62)

c-Myc
IgG

c-Myc

Time   20    0   10   20  (min)

**c**

Control RNAi   Luciferase RNAi   PP2A C RNAi

Blot: anti-

c-Myc

PP2A C

    1      2     3

**Figure 3** Ser 62 phosphate is a substrate for PP2A. (**a**) PP2A dephosphorylates c-Myc at Ser 62. Quiescent REF52 cells were infected with Ad-c-Myc (MOI = 50) together with Ad-Ras$^{Q61L}$ (MOI = 200), maintained in starvation medium and harvested 20 h after infection. c-Myc was immunoprecipitated from cell lysates and incubated with either control buffer (&minus;), 1 μg of purified PP1 (human recombinant) or 0.1 μg of PP2A (bovine kidney), as indicated, at 30 °C for 30 min. Samples were run in triplicate and immunoblotted with the phospho-Ser 62 antibody, the phospho-Thr 58 antibody, or the C-33 c-Myc antibody, as indicated. (**b**) Thr 58 phosphorylation is necessary for PP2A-mediated dephosphorylation of Ser 62. Quiescent REF52 fibroblasts were infected with wild-type Ad-c-Myc or Ad-c-Myc$^{T58A}$ virus (MOI = 200) and maintained in low-serum medium overnight. At 4 h before harvesting, cells were treated with 10 μM MG132 and 100 nM okadaic acid. c-Myc was immunoprecipitated from the cell extracts using the C-33 c-Myc monoclonal antibody. Immunoprecipitated

c-Myc samples were incubated with purified PP2A for the indicated times. Western blot analysis was performed with the phospho-Ser 62 antibody or the C-33 c-Myc antibody. The lower band in the anti-c-Myc blot is IgG. (**c**) Specific inhibition of PP2A by siRNA results in accumulation of c-Myc. 293a cells were transfected with 500 ng CMV-c-Myc and 100 ng CMV-β-gal, along with 100 nM scrambled siRNA (lane 1), siRNA specific for luciferase (lane 2), or pooled siRNAs consisting of four different siRNAs specific for the PP2A C subunit (lane 3). Cells transfected with scrambled siRNA and luciferase siRNA also received 250 ng pGL3-luciferase plasmid. Transfected cells were serum-starved for 48 h and harvested. β-Galactosidase activity was measured to adjust protein load volumes for transfection efficiency. The top half of the western blot was probed with the c-Myc antibody (N262) and the bottom half with the PP2A C subunit antibody. Luciferase assays from lane 1 and 2 extracts are presented in Supplementary Information, Fig. S2.

62 phosphorylation[11,14]. Whereas PP2A effectively dephosphorylated Ser 62 in wild-type c-Myc (Fig. 3b, left), the c-Myc$^{T58A}$ mutant was not dephosphorylated by PP2A (Fig. 3b, right). These results suggest a role for Thr 58 phosphorylation in facilitating removal of Ser 62 phosphate by PP2A.

In addition, accumulation of c-Myc *in vivo* was assessed using RNA interference (RNAi) to inhibit endogenous PP2A activity. Small-interfering RNA (siRNA) directed against the catalytic subunit (C) of PP2A reduced the level of endogenous C subunit, and

coincident with this reduction there was an increase in the level of c-Myc protein (Fig. 3c). In contrast, a scrambled sequence siRNA or an siRNA targeting luciferase had no effect on the level of the C subunit and did not alter c-Myc levels, although the luciferase siRNA did inhibit luciferase activity in this experiment (see Supplementary Information, Fig. S2). Taken together, these results suggest that PP2A contributes to degradation of c-Myc by dephosphorylating Ser 62 and imply that this degradation is dependent on Thr 58 phsophorylation.
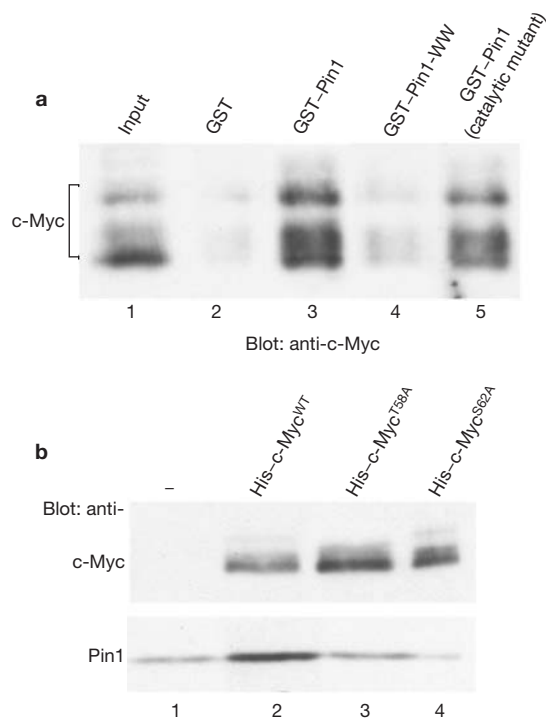
**Figure 4** Pin1 interacts with c-Myc. (**a**) Interaction between c-Myc and Pin1 requires the Pin1 WW domain. Quiescent REF52 fibroblasts were infected with Ad-c-Myc (MOI = 25). Infected cells were maintained in medium with 0.25% serum for 20 h and then harvested in low-stringency buffer. Cell lysates from equal cell counts were incubated with either GST, or GST–Pin1 wild-type or mutant proteins bound to glutathione–sepharose beads, as indicated. Precipitated proteins were washed four times in low-stringency buffer and then released with SDS sample buffer. Samples plus a 10% volume of input lysate were subjected to western blot analysis with the C-33 c-Myc antibody. (**b**) c-Myc and Pin1 interact *in vivo*. 293a cells were transfected with 2 μg pD40-His-c-Myc, pD40-His-c-Myc$^{T58A}$, pD40-His-c-Myc$^{S62A}$ or empty pD40-His vector (–), as indicated, plus 2 μg Ad-Trk-Pin1 and 0.5 μg CMV-β-gal. Cells were serum-starved for 48 h and then harvested by lysis in low-stringency co-immunoprecipitation buffer. Supernatants were normalized for β-galactosidase levels and incubated with nickel–NTA agarose beads at 4 °C for 3–4 h to precipitate His$_6$-tagged c-Myc proteins. Precipitated proteins were washed with co-immunoprecipitation buffer containing 20 mM imidazole and eluted with co-immunoprecipitation buffer containing 250 mM imidazole. Eluted proteins were analysed by western blotting. The upper half of the western blot was probed with anti-Myc (N262) and the bottom half with anti-Pin1, as indicated.

## The Pin1 prolyl isomerase interacts with c-Myc

PP2A may be a conformation-sensitive protein phosphatase, preferring the *trans* configuration of a proline residue adjacent to the phospho-serine or phospho-threonine in substrates such as Cdc25 and Tau[18]. In addition, the phosphorylation-directed prolyl isomerase Pin1 may catalyse the isomerization of proline residues in such phospho-proteins to promote their dephosphorylation by PP2A[19]. Furthermore, the presence of doubly phosphorylated serine/threonine-proline residues, as found in the motifs that comprise the carboxy-terminal domain of RNA polymerase II, increase the binding affinity and catalytic efficacy of Pin1 towards these substrates through recognition of one of the two phosphorylated residues by the Pin1-WW domain[21,22]. These findings led us to test whether c-Myc could bind to Pin1. As an alternative approach, we have used quiescent

REF52 fibroblasts infected with Ad-Myc, made extracts from these cells and incubated them with glutathione S-transferase (GST) alone, GST–Pin1 or GST–Pin1 bearing point mutations in either the WW domain or the catalytic domain[30] immobilized on glutathione–agarose beads. c-Myc bound preferentially to wild-type GST–Pin1 beads, compared with GST alone (Fig. 4a, compare lanes 2 and 3). c-Myc interacted poorly with the Pin1$^{W33A}$ WW mutant (Fig. 4a, lane 4), but bound the Pin1$^{C109A}$ catalytic mutant with similar efficacy to wild-type Pin1 (Fig. 4a, compare lanes 3 and 5).

To investigate the role of Thr 58 and Ser 62 phosphorylation in the Pin1–Myc interaction, the ability of wild-type c-Myc, c-Myc$^{T58A}$ or c-Myc$^{S62A}$ mutants[11] to interact with Pin1 *in vivo* was examined. 293a cells were transfected with vectors to express His$_6$-tagged wild-type or mutant c-Myc proteins. Cells were lysed and His$_6$–Myc proteins were selected on nickel–NTA beads. Bound material was eluted with imidizole and then analysed by western blotting. Pin1 was recovered in association with wild-type c-Myc (Fig. 4b, compare lanes 1 and 2), but a reduced interaction was detected with either c-Myc$^{T58A}$ or c-Myc$^{S62A}$ (Fig. 4b, lanes 3 and 4). As c-Myc$^{T58A}$ remains highly phosphorylated on Ser 62, whereas c-Myc$^{S62A}$ lacks phosphorylation at either Thr 58 or Ser 62 (refs 11–14), these results suggest that the phosphorylation of c-Myc at Thr 58 is important for Pin1 binding.

## Pin1 facilitates c-Myc protein degradation

We next examined whether Pin1 expression affected the level of c-Myc protein by utilizing primary mouse embryo fibroblasts (MEFs) derived from *Pin1*$^{-/-}$ embryos. Heterozygous *Pin1* mice, maintained in an iso-genic C57BL/6J background, were bred, and sibling 13.5-day embryos were used to prepare primary MEFs with either *+/+* or *−/−* genotypes[23]. Substantially more c-Myc protein accumulated in *Pin1*$^{-/-}$ cells, compared with wild-type MEFs after Ad-Myc infection (Fig. 5a, compare lanes 1 and 2). This increase in c-Myc levels was caused by the absence of Pin1, as re-introduction of Pin1 (using Ad-Pin1) into the *Pin1*$^{-/-}$ MEFs reduced c-Myc levels to those detected in wild-type MEFs (Fig. 5a, compare lanes 1 and 3). Furthermore, a pulse-chase experiment demonstrated that the increased accumulation of c-Myc in the absence of Pin1 was associated with an increased half-life of c-Myc in *Pin1*$^{-/-}$ MEFs (Fig. 5b). Moreover, additional pulse-chase assays demonstrated that re-introduction of Pin1 into *Pin*$^{-/-}$ cells reduced the half-life of c-Myc in these cells (see Supplementary Information, Fig. S3).

The accumulation of c-Myc observed in the absence of Pin1 was not significantly affected by inhibition of PP2A through expression of small T antigen (Fig. 5c, lanes 2 and 4), when compared with the effects of small T antigen expression on c-Myc in *Pin1*$^{+/+}$ cells (Fig. 5c, lanes 1 and 3). This result suggests a similar role for the actions of Pin1 and PP2A on c-Myc. Moreover, the enhancement of wild-type c-Myc accumulation in the absence of Pin1 (Fig. 5d, top) was not observed for the c-Myc$^{T58A}$ mutant (Fig. 5d, bottom), consistent with the observation that this mutant shows reduced binding to Pin1 and displays increased Ser 62 phosphorylation[11]. Taken together, these results suggest a complementary role for Pin1 and PP2A in controlling c-Myc turnover.

## Pin1 ensures transient accumulation of c-Myc after growth stimulation

Given the role of Pin1 in the degradation of c-Myc, we investigated the contribution of Pin1 to the normal regulation of c-Myc levels after growth stimulation. Wild-type or *Pin1*$^{-/-}$ primary MEFs were density arrested and then plated into low-serum medium for 24 h. The quiescent MEFs were then stimulated with 20% foetal calf serum and endogenous c-Myc protein levels were determined by western blotting at 0, 3, 6 and 24 h after serum addition. Endogenous c-Myc in wild-type
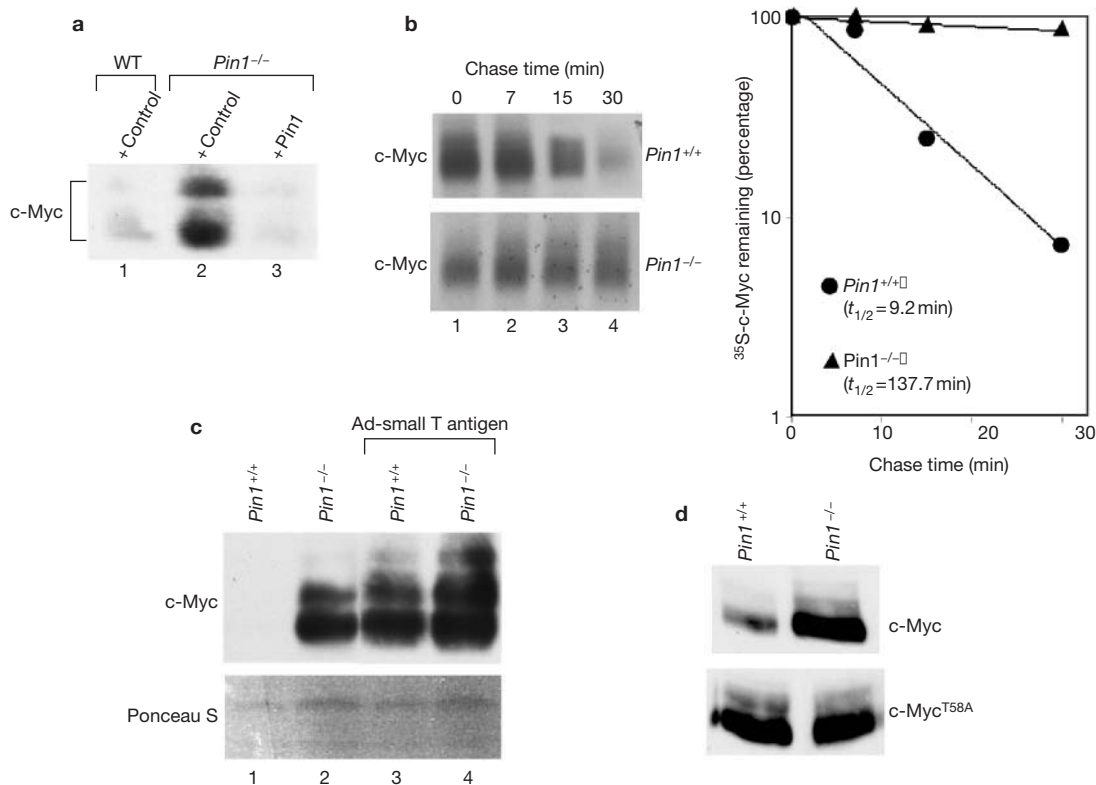
**Figure 5** Accumulation of c-Myc is regulated by Pin1. (**a**) c-Myc levels are elevated in *Pin1*^{−/−} cells. Passage-3 primary MEFs from siblings with either wild-type (WT) or *Pin1*^{−/−} genotypes were made quiescent by serum starvation for 48 h and then infected with Ad-c-Myc (MOI = 100) together with either Ad-GFP (control) or Ad-Pin1 (both at MOI = 400). Infected cells were maintained in medium with 0.25% serum for 20 h and extracts prepared from an equal number of cells for each condition were subjected to western blot analysis with the C-33 c-Myc antibody. (**b**) c-Myc half-life is increased in the absence of Pin1. Quiescent primary *Pin1*^{+/+} or *Pin1*^{−/−}MEFs were infected with Ad-c-Myc (MOI = 100) and maintained in low-serum medium. At 18 h after infection, cells were labelled with $^{35}$S-methionine/cysteine for 30 min and chased in medium with 0.20% serum containing excess unlabelled methionine and cysteine for the indicated times. Labelled c-Myc was immunoprecipitated from equal cell numbers at each time, analysed by SDS–PAGE and then quantified with a phosphorimager. Best-fit exponential lines are shown for c-Myc expressed in *Pin1*^{+/+} and *Pin1*^{−/−} MEFs. (**c**) c-Myc protein levels in *Pin1*^{−/−} MEFS are not affected by small T antigen. Quiescent primary *Pin1*^{+/+} or *Pin1*^{−/−}MEFs were infected with Ad-c-Myc (MOI = 100) and either Ad-GFP (control; MOI = 400) or Ad-small T (MOI = 400). Infected cells were treated as described in **a**. Equal protein loading was confirmed by Ponceau S staining and measurement of a reference band. (**d**) The c-Myc[T58A] mutant is not affected by deletion of Pin1. Quiescent primary *Pin1*^{+/+} or *Pin1*^{−/−}MEFs (passage 3) were infected with Ad-c-Myc or Ad-c-Myc[T58A] (MOI = 100) and treated as described in **a**.

MEFs exhibits a typical temporal pattern[11]: that is, a low level in quiescent cells (Fig. 6, lane 1), a peak accumulation at 3 h (Fig. 6, lane 2), a decline at 6 h (Fig. 6, lane 3) and a further decrease to a basal steady-state level by 24 h (Fig. 6, lane 4). Quiescent MEFs lacking Pin1 also showed undetectable levels of c-Myc protein before serum stimulation, because of very low levels of c-*myc* gene transcription in growth-arrested cells (Fig. 6, lane 5). Endogenous c-Myc was induced at 3 h after serum stimulation in *Pin1*^{−/−} cells (Fig. 6, lane 6). In contrast to wild-type MEFs, however, c-Myc levels increased continuously with time, reaching maximal levels at 24 h after serum stimulation (Fig. 6, lane 8). This result is very similar to that resulting from inhibition of GSK-3β activity[11] and is consistent with stabilization of c-Myc in the *Pin1*^{−/−} cells. In addition, the increased levels of c-Myc in the *Pin1*^{−/−} cells correlated with enhanced Ser 62 phosphorylation (Fig. 6, second panel). This is particularly notable at the 6-h time point, when total levels of c-Myc are largely equivalent between wild-type and *Pin1*^{−/−} MEFs, but when phospho-Ser 62 levels are much higher in the *Pin1*^{−/−} MEFs (Fig. 6, compare lanes 3 and 7). This result was also supported by treatment of wild-type MEFs with okadaic acid, which also increased Ser 62 phosphorylation and prolonged c-Myc expression in response

to growth stimulation (data not shown). Examination of Pin1 levels in the same samples demonstrates that Pin1 expression in wild-type MEFs is not affected by serum stimulation (Fig. 6). These results suggest that one way in which Pin1 promotes a decrease in c-Myc levels is through enhancing Ser 62 dephosphorylation.

## Control of c-Myc phosphorylation affects c-Myc transactivation capacity

A primary function of c-Myc is to activate transcription of a large number of target genes encoding proteins important for cell growth. To assess the functional significance of the regulation of c-Myc phosphorylation and turnover by PP2A, the transcriptional activation capacity of c-Myc was assessed in the absence or presence of small T antigen. Previously, we demonstrated a role for c-Myc in activation of the E2F2 promoter that is dependent on the presence of c-Myc-binding sites (E-box elements) in the promoter[24]. To examine how controlling c-Myc degradation affects its function, we reduced the level of Ad-Myc sufficiently to minimally activate the E2F2 promoter (Fig. 7; E2F2-Luc, compare control with c-Myc). We also observed only minimal effects on the activity of the E2F2 promoter as a result of expressing small T
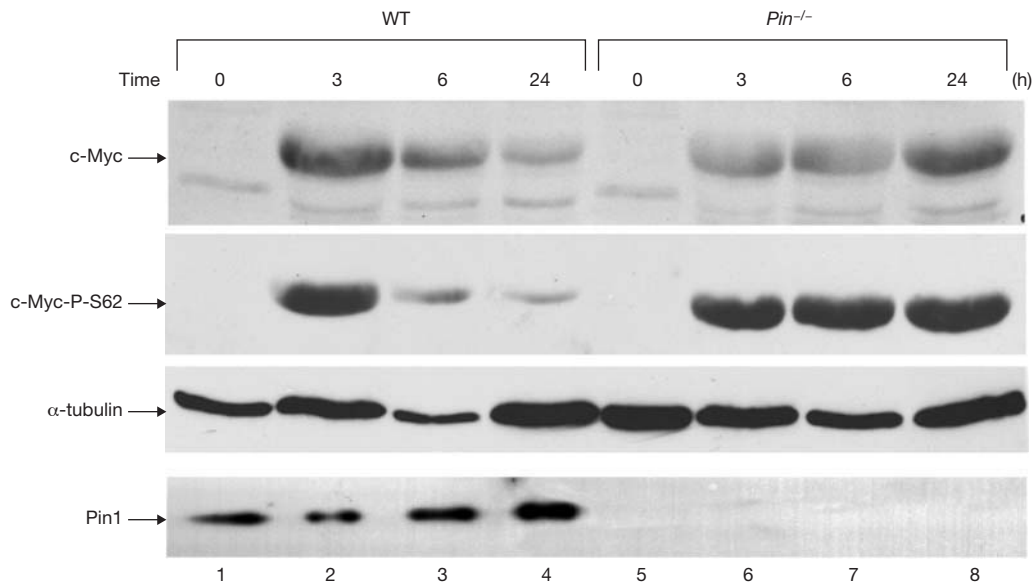
**Figure 6** Pin1 is essential for normal control of c-Myc accumulation. Accumulation of endogenous c-Myc is prolonged in $Pin1^{-/-}$ fibroblasts and shows increased Ser 62 phosphorylation. Primary $Pin1^{+/+}$ or $Pin1^{-/-}$ MEFs (passage 2) were density growth-arrested, plated at low density into medium with 0.25% serum and incubated for 24 h. These quiescent cells were then stimulated by the addition of 20% FCS for the indicated times. Equal cell counts from serum-starved (0 h) or stimulated (3, 6, and 24 h) cells were harvested and subjected to western blot analysis with the mouse monoclonal C33 c-Myc antibody (top). The blot was stripped and re-probed with the rabbit polyclonal phosphoSer62 antibody (second panel) or the $\alpha$-tubulin antibody (third panel). Samples were then re-run and probed with the anti-Pin1 antibody (bottom).

antigen. In contrast, co-expression of small T antigen and c-Myc resulted in a substantial increase in E2F2 promoter activity, and this activation was dependent on the presence of intact c-Myc-binding sites in the promoter (Fig. 7; E2F2(-Ebox)-Luc, c-Myc + tAg). We conclude that the control of c-Myc phosphorylation not only regulates the stability of c-Myc, but also has a direct impact on c-Myc function.

**Stabilized c-Myc can replace small T antigen in transformation of human cells**

Human fibroblasts can be transformed by a combination of Ras, telomerase and SV40 large T and small T antigens[25]. Given the role of small T antigen in regulating PP2A, together with the role for PP2A in mediating the destabilization of c-Myc, we evaluated whether a stabilized form of c-Myc could replace small T antigen as a necessary requirement for transformation and tumorigenesis. Human embryonic kidney (HEK) cells expressing large T antigen, the catalytic subunit of human telomerase (hTERT) and Ras$^{G12V}$ (hereby referred to as HEK-TER cells) cannot grow in soft agar (a measure of transformed cell growth) or form tumours in mice, unless small T antigen is introduced[26]. We ectopically expressed the stable c-Myc$^{T58A}$ mutant in the HEK-TER cells or independently expressed small T antigen (as a positive control), wild-type c-Myc or c-Myc$^{S62A}$ (which cannot be phosphorylated and consequently is rapidly degraded[11]) as negative controls, and developed cell populations that expressed each of the four proteins. These cell populations were then assayed for anchorage-independent growth in soft agar, the best *in vitro* corollary to tumorigenic growth *in vivo*. Addition of small T antigen promoted aggressive transformed growth, as previously reported[26], whereas cells expressing wild-type c-Myc or c-Myc$^{S62A}$ failed to grow in an anchorage-independent manner (Fig. 8a). However, the cells expressing stabilized c-Myc$^{T58A}$ protein grew in soft agar identically to cells expressing small T antigen (Fig. 8a). Similar transformation trends for the c-Myc$^{S62A}$ and

c-Myc$^{T58A}$ mutants have been reported previously in rodent cells[12,13]. To confirm our result, mixed populations were examined, as well as clonally isolated cell lines expressing c-Myc$^{T58A}$. All these cell lines exhibited growth in soft agar, whereas none of the wild-type c-Myc populations were able to grow (see Supplementary Information, Fig. S4). Finally, c-Myc$^{T58A}$ was tested in a second human cell type — BJ fibroblasts also containing small T antigen, hTERT and Ras$^{G12V}$ — and compared with small T antigen as a positive control or vector alone as a negative control. c-Myc$^{T58A}$-expressing cells grew as well as the small-T-antigen-positive control cells (Fig. 8a). Thus, a stable, and therefore oncogenic, c-Myc can functionally replace small T antigen in transformed cell growth of human cells expressing a defined set of genes.

We then questioned whether c-Myc$^{T58A}$ could also replace small T antigen in the most stringent test of human oncogenesis: *in vivo* tumour growth. The four HEK-TER cell populations described above were assayed for tumour growth in immuno-compromised mice. As previously observed, small T antigen was essential for primary human cells expressing large T antigen, hTERT and Ras$^{G12V}$ to form tumours (Fig. 8b). Consistent with soft agar assays, mice injected with cells expressing wild-type c-Myc or the c-Myc$^{S62A}$ mutant completely failed to exhibit tumour growth for up to 35 days, which is three times longer than it takes positive control cells expressing small T antigen to form tumours. Strikingly, cells expressing c-Myc$^{T58A}$ formed tumours in an almost identical manner to cells expressing small T antigen (Fig. 8b). On the basis of these results, we conclude that the oncogenic transformation of human cells can be accomplished by the combined action of large T antigen, Ras, telomerase, and an activated, stabilized, c-Myc. Thus, at least one functional consequence of c-Myc stabilization in human cells is to sensitize the cells to transformation and tumorigenesis.

**DISCUSSION**

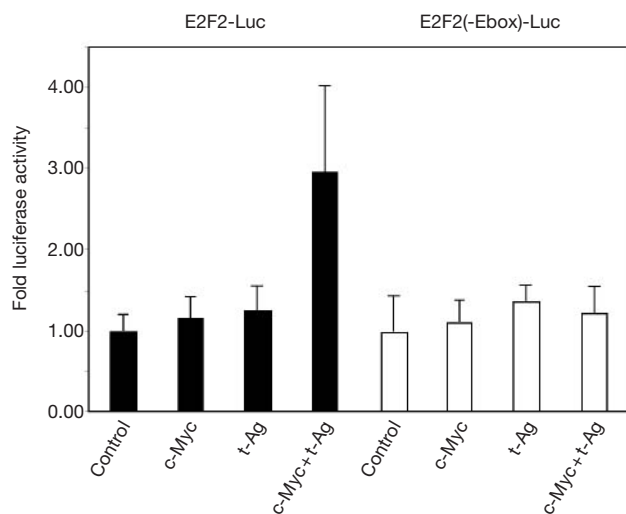Although an increase in protein levels could be achieved in principle

**Figure 7** Small T antigen enhances the transactivation function of c-Myc. REF52 fibroblasts were transfected by calcium phosphate co-precipitation with either wild-type E2F2-luciferase construct (E2F2-Luc) or mutant E2F2-luciferase construct containing point mutations in the three E-box c-Myc-binding sites (which eliminates c-Myc binding to the E2F2 promoter; E2F2(-Ebox)-Luc), plus CMV-β-gal as an internal control. After 16 h, cells were placed in DMEM containing 0.25% FCS for 48 h. Transfected cells were then infected with Ad-GFP (MOI = 200) (Ctrl), Ad-c-Myc (MOI = 100), Ad-small T antigen (MOI = 100) or Ad-c-Myc (MOI = 100) + Ad-small T antigen (MOI = 100), as indicated. Cells were harvested 20 h after infection, and luciferase and β-galactosidase activities were measured. Luciferase activity was normalized to β-galactosidase activity, and data are presented as fold activation over control. Transfections were performed in two separate experiments and the mean fold induction from three separate data points per condition is shown, with error bars indicating standard deviations.

through transcriptional activation coupled with a short half-life of the protein, the action of Ras provides a mechanism to amplify c-Myc accumulation by stabilizing this otherwise short-lived protein. We suggest that the role of the inter-related phosphorylation and dephosphorylation events is to ensure that Ras-mediated amplification of c-Myc protein levels is indeed transient and self-limited. The Raf–MEK–ERK pathway stabilizes c-Myc by enhancing Ser 62 phosphorylation, and the PI(3)K/Akt pathway prevents the subsequent phosphorylation of c-Myc at Thr 58 by inhibiting GSK-3β. However, as Ras activity declines after cessation of the growth stimulus, PI(3)K and Akt activities also decline, resulting in reactivation of GSK-3β and phosphorylation of c-Myc on Thr 58. Our data suggest that phosphorylation of Thr 58 is important for recognition of c-Myc by the Pin1 prolyl isomerase, the actions of which facilitate c-Myc dephosphorylation at Ser 62 by PP2A, which then promotes c-Myc turnover by the ubiquitin-proteasome pathway. Thus, the very mechanism that stabilizes and amplifies c-Myc accumulation — c-Myc phosphorylation at Ser 62 — also triggers the subsequent phosphorylation at Thr 58 and the series of events that culminate in degradation of c-Myc.

Considerable evidence suggests that Thr 58 phosphorylation is critical for ensuring the transient and timely degradation of c-Myc. All v-*myc* genes recovered in transforming retroviruses harbour a mutation at Thr 58 (ref 27). Similarly, a large number of c-*myc* genes amplified in Burkitt's lymphoma carry a mutation at Thr 58, as well as in other residues between amino acids 57 and 63 (refs 12, 13, 16, 17 and 28). Assay of these mutants generally demonstrates their increased oncogenic potential in both cell transformation assays and animals[12,13,29]. In

conjunction with other studies, our results confirm that mutation of c-Myc at Thr 58 increases the stability of c-Myc, highlighting a key role for this phosphorylation site in controlling c-Myc degradation[11,16,17]. These results are consistent with a model in which the Pin1 WW domain binds to c-Myc phosphorylated at Thr 58 in a manner that could result in a conformational change that makes phospho-Ser 62 an ideal site for dephosphorylation by PP2A. Our additional finding that a stabilized c-Myc protein can completely substitute for small T antigen by collaborating with Ras, hTERT and small T antigen in the transformation and oncogenic conversion of primary human cells, whereas wild-type c-Myc cannot do so, establishes the importance of c-Myc stability in human cancer.  □

## METHODS

**Antibodies.** The C-33 and N262 c-Myc antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA). The ubiquitin antibody is from Zymed (South San Francisco, CA). The Thr 58 phosphospecific antibody is from Upstate Cell Signaling (Waltham, MA). The Ser 62 phosphospecific antiserum was prepared as described previously[11]. The Pin1 antibody was prepared as described previously[30]. The α-tubulin antibody is from Sigma (St Louis, MO). The PP2A catalytic unit antibody is from BD Biosciences (San Jose, CA).

**Plasmids.** CMV-Myc, CMV-β-gal, E2F2-Luc, and E2F2(-Ebox3)-Luc plasmids have been previously described[24]. His6-tagged c-Myc was created by PCR amplification of the coding sequence for wild-type murine c-Myc2 from the CMV-Myc plasmid with a CACC 5′ extension added to the 5′ primer for cloning into the TOPO entry clone from Invitrogen (Carlsbad, CA). This sequence was then moved into the pDEST-40 mammalian expression vector using Gateway technology (Invitrogen), creating pD40-His-c-Myc. His6-tagged c-Myc[T58A] and His6-tagged c-Myc[S62A] were constructed similarly, except that PCR amplification was from the CMV-Myc[T58A] and CMV-Myc[S62A] plasmids previously described[11], creating pD40-His-c-Myc(T58A) and pD40-His-c-Myc[S62A]. The Ad-Trk-Pin1 plasmid was constructed as described below.

**Cells.** REF52 cells were grown in DMEM containing 5% foetal calf serum (FCS) and 5% calf serum (CS). To bring REF52 cells to quiescence, cells were plated at ~3,500 cells cm⁻² and incubated overnight. The next day the culture medium was replaced with DMEM containing 0.25% serum (FCS/CS) and cells were incubated for an additional 48 h. Primary MEFs were grown in DMEM containing 15% heat-inactivated FCS. MEFs were made quiescent either by plating passage-3 MEFs at a density of approximately 2,000 cells cm⁻² and the next day replacing the medium with DMEM containing 0.2% heat-inactivated FCS for 48 h, or by allowing passage-2 MEFs to grow for 24 h after they had reached confluence and then plating them directly into DMEM/0.2% serum at 4,000 cells cm⁻² for 24 h. Where indicated, cells were serum-stimulated by adding FCS directly to the starvation media to a final concentration of 20%. 293 cells obtained from the American Type Culture Collection were grown in DMEM containing 10% FCS. For serum-starvation conditions, exponentially growing 293 cells were washed with DMEM, refed with DMEM containing 2% FCS and incubated for 48 h.

**Viruses.** Stocks of purified virus were created as described previously[31]. Viral titres (MOI) were determined in 293 cells using an indirect immunofluorescence microscopy assay specific for the viral 72K E2 gene product, as described[32]. Experimental comparisons demonstrate that REF52 fibroblasts are approximately 400-fold less susceptible to infection than 293 cells, and MEFs are approximately 1,200-fold less susceptible. Quiescent REF52 or MEFs were infected with virus by incubation in DMEM containing 20 mM Hepes at pH 7.2 for 75 min at 37 °C at a cell-to-volume ratio of 5 × 10⁵ cells ml⁻¹. After infection, four volumes of DMEM/0.25% (REF52) or 0.2% (MEF) serum were added to each plate and the cells were further incubated at 37 °C for 18–20 h before harvest and analysis. The construction of Ad-c-Myc, Ad-c-Myc[T58A] and Ad-c-Myc[S62A] has been described[11]. Ad-GFP has been described previously[33]. Ad small T antigen was constructed by digesting the pCEP small T plasmid with *Sma*I and *Hind*III, and ligating this fragment containing the SV40 small T antigen cDNA into the *Hind*III/*Eco*RV sites in the AdTrack-CMV plasmid used to
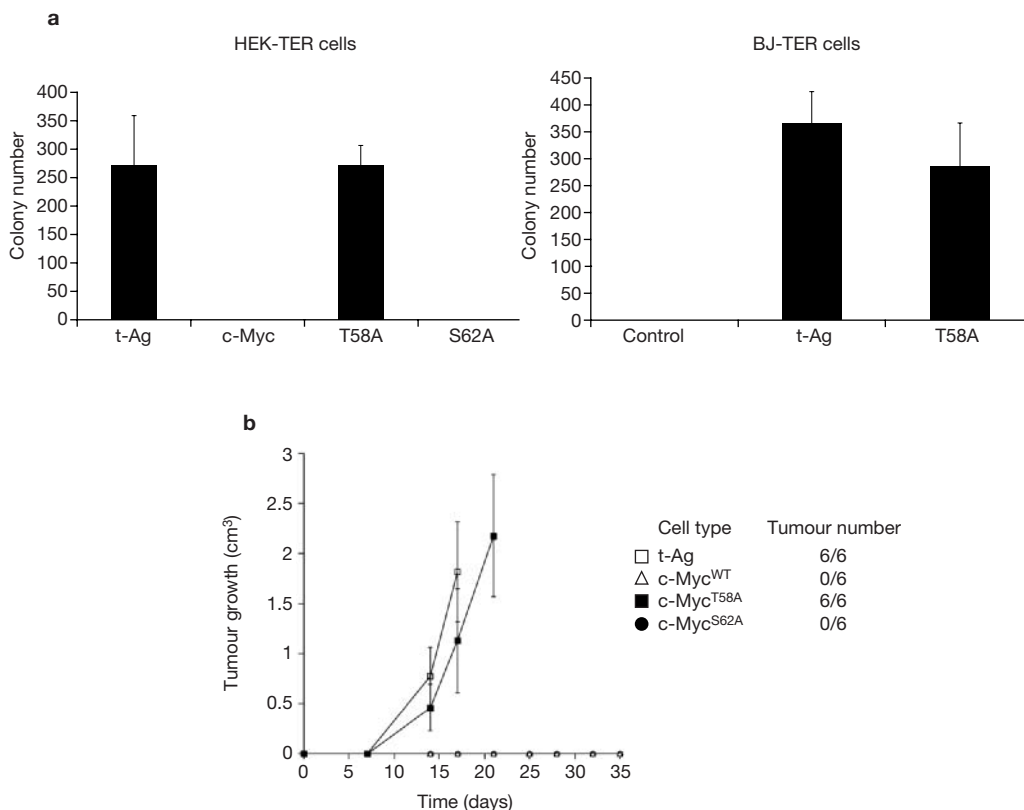
**a**



HEK-TER cells

BJ-TER cells

**b**



| Cell type | Tumour number |
| --- | --- |
| □ t-Ag | 6/6 |
| △ c-Myc$^{WT}$ | 0/6 |
| ■ c-Myc$^{T58A}$ | 6/6 |
| ● c-Myc$^{S62A}$ | 0/6 |

**Figure 8** Stabilized c-Myc cooperates with Ras and telomerase to transform human fibroblasts. (**a**) c-Myc$^{T58A}$ has transforming properties. Anchorage-independent growth of the indicated cells stably infected with retroviruses encoding small T antigen, c-Myc, c-Myc$^{S62A}$ or c-Myc$^{T58A}$, calculated from the average number of colonies observed in three plates. (**b**) c-Myc$^{T58A}$ replaces small T antigen function in tumorigenesis assays. Mean ± standard error of tumour volumes (cm$^3$) from six mice injected with HEK cells expressing hTERT, T-ag, Ras$^{G12V}$ and either small T antigen, c-Myc, c-Myc$^{S62A}$ or c-Myc$^{T58A}$. $n = 6$ for each injection.

construct recombinant adenoviruses as described[34]. Ad-Pin was similarly constructed by digesting the pET28-Pin1 plasmid with *Bam*HI and *Xho*I and then ligating this fragment containing the human Pin1 cDNA into the *Bgl*II/*Xho*I sites in AdTrack-CMV, creating the plasmid Ad-Trk-Pin1 used to construct the Ad-Pin1 virus.

**Detection of ubiquitinated Myc.** Cells were washed once with cold PBS and lysed with RIPA buffer containing standard protease and phosphatase inhibitors, as described[10], with the addition of the de-ubiquitinase inhibitor 5 mM N-ethylmaleimide (NEM; Sigma) at a cell-to-volume ratio of $1 \times 10^6$ cells ml$^{-1}$. Ubiquitinated proteins were immunoprecipitated from cell lysates with an anti-ubiquitin antibody from Zymed. After precipitation with the protein A/protein G–agarose beads, immunoprecipitates were washed once with low-stringency buffer containing PBS, 0.1% NP40 (Sigma) and all inhibitors used with the RIPA buffer. Immunoprecipitated proteins were then separated by 10% SDS–PAGE, blotted to immobilon-P membrane and detected with the indicated c-Myc antibodies.

**Western blotting.** Cells were harvested by scraping into hot 1× SDS sample buffer at a cell-to-volume ratio of $2.5 \times 10^6$ cells ml$^{-1}$. Protein concentrations were determined for normalization purposes using the BCA solutions (Sigma) according to the manufacturer's instructions. Equal protein for each sample was separated by SDS–PAGE and blotted to Immobilon-P membrane (Millipore, Billerica, MA). Primary antibodies were in 5% non-fat milk, PBS, 0.1% Tween20, except anti-phospho-Ser 62 antibody which was in 5% heat-inactivated horse serum/5% BSA, PBS, 0.1% Tween20. Primary antibodies were detected with HRP-conjugated secondary antibodies using the ECL reagents from Amersham according to the manufacturer's instructions.

**Immunoprecipitations.** Cells were harvested by washing once in cold PBS, followed by lysis in cold c-Myc antibody lysis buffer containing protease and phosphatase inhibitors as previously described[10]. c-Myc proteins were immunoprecipitated from equal numbers of cells for each sample at a cell-to-volume ratio of $1 \times 10^6$ cells ml$^{-1}$ lysis buffer using a 1:100 dilution of the c-Myc monoclonal antibody C-33 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody incubation was performed at 4 °C for 14–18 h followed by immuno-precipitation with protein A/protein G–agarose beads and washing with lysis buffer containing protease and phosphatase inhibitors.

**Pulse-chase assays.** MEFs or REFs were pre-starved for methionine and cysteine by replacing the culture medium with DMEM lacking L-Methionine and L-Cysteine for 15 min. Cells were labelled *in vivo* with $^{35}$S-methionine/cysteine EXPRESS protein labelling mix from Perkin Elmer (Boston, MA) using 500 μCi ml$^{-1}$ at a cell-to-volume ratio of $7.5 \times 10^5$ cells ml$^{-1}$ for 20–30 min. After labelling, cells were immediately washed once with DMEM containing 5 mM L-methionine, 3 mM L-cysteine and 0.2% serum, and then incubated in the same media for the indicated chase times. Cells were harvested and labelled c-Myc proteins were immunoprecipitated as described above. Labelled c-Myc was visualized by autoradiography and quantified with a phosphorimager.

**Myc–GST–Pin1 interaction assays.** GST and GST–Pin1 fusion proteins with either wild-type *Xenopus laevis* Pin1, a *X. laevis* Pin1 WW-domain mutant with substitutions at W11A and W34A, or a *X. laevis* Pin1 catalytic-deficient mutant with a substitution at C109A were prepared as described[30]. The GST and GST–Pin1 recombinant proteins were bound to glutathione–agarose beads as follows: 10 μg of recombinant protein were incubated with 25 μl of a 50% slurry of glutathione–agarose beads in 500 μl of a low-stringency buffer containing 1× PBS, 0.5% NP40 and 5% glycerol with standard protease and phosphatase

inhibitors[10]. Binding was performed at 4 °C for 1.5 h. GST beads were then washed four times in 600 μl of low-stringency buffer. REF52 cells infected with Ad-c-Myc were lysed in low-stringency buffer at a cell-to-volume ratio of $5 \times 10^5$ cells ml$^{-1}$. 500 μl of cell lysate containing c-Myc was then incubated with the various GST–Pin1 beads for 4 h at 4 °C. Samples were then washed three times in 600 μl of low-stringency buffer and precipitated proteins were subjected to western blot analysis.

**Co-precipitation of Myc and Pin1.** 293 cells were transfected with expression plasmids for wild-type or mutant His$_6$-tagged c-Myc proteins, Pin1 and β-galactosidase. Cells were lysed in co-immunoprecipitation buffer (50mM Hepes at pH 7.2, 125 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Tween-20 and 12.5% glycerol) containing protease and phosphatase inhibitors. Extracts were normalized for transfection efficiency by β-galactosidase assay and incubated with nickel–NTA–agarose beads (Qiagen, Valencia, CA) for 3–4 h at 4 °C followed by three washes in co-immunoprecipitation buffer containing protease and phosphatase inhibitors and 20 mM imidazole. Precipitated proteins were released with co-immunoprecipitation buffer containing 250 mM imidazole and then analysed by western blotting.

**Primary wild-type and Pin1$^{-/-}$ MEFs.** Pin1$^{-/-}$ mice were generated as previously described[35]. The *Pin1* gene deletion was transferred into an isogenic C57Bl6 background using marker-assisted speed congenic breeding by Jackson Laboratory (Bar Harbor, ME). *Pin1$^{+/+}$* and *Pin1$^{-/-}$* embryonic fibroblasts were isolated from the isogenic strain as detailed previously[36] and processed as described above.

**Assay of endogenous c-Myc.** Asynchronous or quiescent REF52 cells were infected with Ad-small T antigen or control Ad-GFP, and endogenous c-Myc was immunoprecipitated as described under Immunoprecipitations, except that cells were lysed in antibody lysis buffer at $2 \times 10^6$ cells ml$^{-1}$ and precipitates were not washed, but loaded directly onto 8% gels and fractionated by SDS–PAGE. Endogenous c-Myc from primary MEFs was assayed after cells were density growth-arrested in DMEM containing 15% heat-inactivated FCS for 24 h after they reached confluence, before& plating at ~4,000 cells cm$^{-2}$ in DMEM containing 0.2% heat-inactivated FCS. After 24 h, cells were serum-stimulated by adding FCS to 20% volume. Samples were then scraped into hot SDS sample buffer at the indicated times and analysed by western blotting as described above.

**Transfections.** REF52 fibroblasts were transfected using calcium phosphate co-precipitation, as previously described[24]. Cells were plated the day before transfection in 100-mm tissue culture dishes at ~3,500cells cm$^{-2}$. Cells were exposed to the CaPO$_4$–DNA precipitate containing 10–20 μg of total DNA for 18–20 h. Transfected cells were washed once with DMEM and placed into starve media (DMEM containing 0.25% FCS) for 48 h. Luciferase activity was measured using a Promega (Madison, WI) luciferase assay system kit, as specified by the manufacturer, and a Berthold luminometer (Bundoora, Australia). β-galactosidase activity was measured as described previously[24]. 293a cells were plated at ~5,000 cells cm$^{-2}$ into tissue culture dishes and incubated overnight. Cells at 60–80% confluency were then transfected with Perfectin (Gene Therapy Systems, San Diego, CA) or TransIT-TKO (Mirus Co., Madison, WI) at a 4:1 ratio of lipid to DNA, in low-serum medium (2% FCS) according to the manufacturer's specifications. Transfected cells were maintained in DMEM containing 2% FCS and harvested 48 h after transfection.

**RNAi experiments.** 293 cells were transfected with expression vectors for c-Myc, β-galactosidase, and Luciferase, along with either pooled siRNAs directed against the PP2A C subunit (Dharmacon, Lafayette, CO) or control siRNAs including a scrambled siRNA or siRNA directed against luciferase from the siSTARTER Luciferase Kit (Dharmacon) using TransIT-TKO (Mirus Co.). Cells were collected 48 h after transfection and analysed for β-galactosidase activity as previously described. Both luciferase activity and protein load volumes for western blot analysis were adjusted for transfection efficiency on the basis of β-galactosidase activity.

**Transformation and tumorigenesis assays.** The retroviral constructs containing c-Myc, c-Myc$^{T58A}$, and c-Myc$^{S62A}$ were created by PCR amplification of cDNA from the pRC-CMV vector[37] using T7 and SP6 universal primers with *Bam*HI

and *Eco*RI sites engineered onto the ends. The DNAs were then ligated into the pWZL-Blast vector digested with the same enzymes to create pWZL-Blast c-Myc, c-Myc$^{T58A}$ and c-Myc$^{S62A}$. The HEK and BJ cell lines containing hTERT, Ras, large T antigen and small T antigen in varying combinations were created by retroviral transduction of parental cells as previously described[26]. The subsequent cell lines containing the various c-Myc constructs were generated by amphotrophic retroviral infection and polyclonal selection with 2.5 μg ml blasticidin S (EMD Biosciences, San Diego, CA).

The ability of cells to undergo anchorage-independent growth was tested as previously described[38]. Briefly, $5 \times 10^4$ cells were plated in 0.3% agar for 12 days (HEK cells) or 21 days (BJ cells) and assessed for colony growth in soft agar. The mean and standard deviation represents two independent experiments performed in triplicate. Tumour formation in immunodeficient mice was determined by sub-cutaneous injection of $1 \times 10^7$ cells per animal and was represented as the number of tumours/number of animals (1 injection site per animal).

*Note: Supplementary Information is available on the Nature Cell Biology website.*

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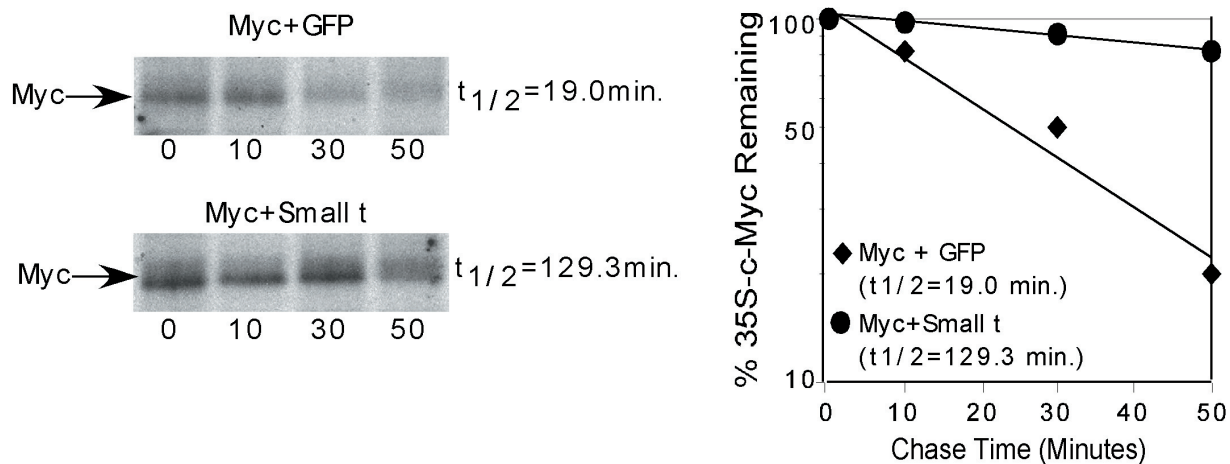
nature
cell biology



**Figure S1.** SV40 small t increases the half life of Myc. Quiescent REF52 fibroblasts were infected with Ad-Myc (MOI=50) and either Ad-GFP (MOI=200) or Ad-Small t (MOI=200). Cells were maintained in medium with 0.25% serum and 18 hr post infection labeled with $^{35}$S-Methionine/Cysteine for 30 min and chased in medium with 0.25% serum containing excess unlabeled Methionine and Cysteine for the indicated times. Labeled Myc was immunoprecipitated from equal cell numbers at each time point, analyzed by SDS-PAGE and quantitated by phosphorimager. Best-fit exponential lines calculated by Excel are shown for Myc+GFP (diamonds) and Myc+Small t (circles).
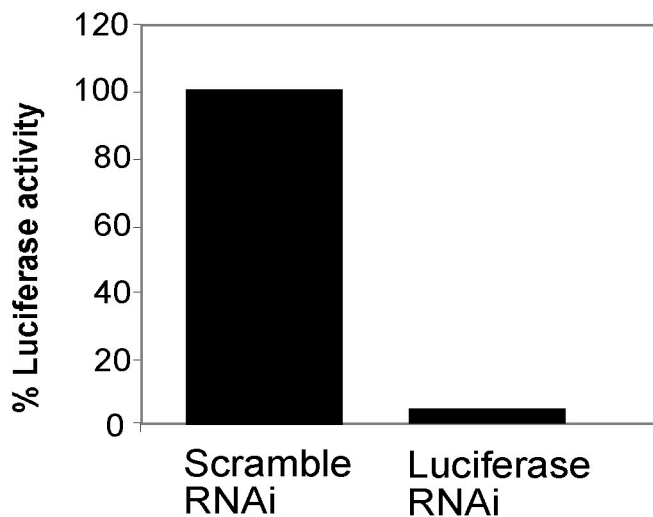


**Figure S2.** Luciferase activity is knocked down in cells transfected with luciferase siRNA. 293a cells were transfected with 500ng CMV-c-Myc, 100ng CMV-β-gal, and 250ng pGL3-luciferase (Dharmacon) along with 100nM of either scrambled siRNA or luciferase specific siRNA (both from Dharmacon). Transfected cells were serum starved for 48 hr and harvested. Luciferase activity was analyzed and adjusted for β-gal activity.
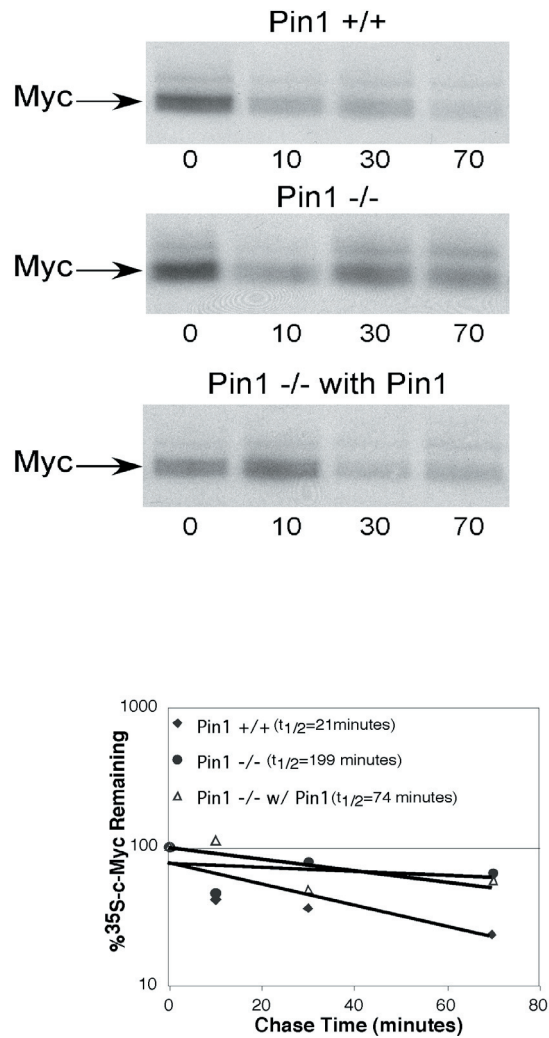
Figure S3. Pin1 decreases the half life of Myc. Quiescent primary MEFs, either wild-type or Pin1-/-, were infected with Ad-Myc (MOI=100) and either Ad-GFP (MOI=300) (upper two panels) or Ad-Pin1 (MOI=300) (lower panel). Cells were maintained in medium with 0.20% serum for 18 hr and then subjected to pulse-chase analysis as described for Supplemental Figure 1. Labeled Myc was analyzed and graphed as described for Supplemental Figure 1 to obtain half-life measurements as shown.
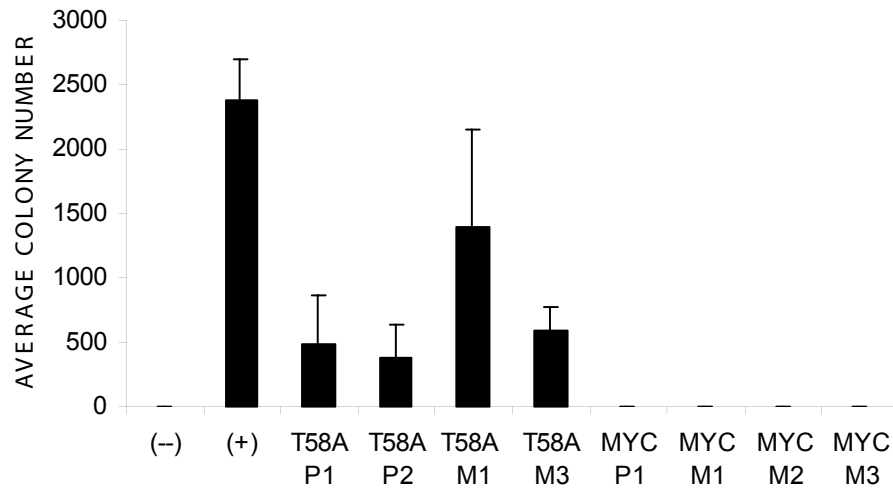
Figure S4. Myc[T58A] is selective for transformation. Anchorage-independent growth of mixed (designated as P) and clonally (designated as M) isolated cell populations, calculated from the average number of colonies observed in 3 plates as previously described.