

Regulation of translation factors eIF4GI and 4E-BP1 during recovery of protein synthesis from inhibition by p53

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Activation of the tumour suppressor protein p53 rapidly inhibits protein synthesis. This is associated with dephosphorylation and cleavage of initiation factor eIF4GI and the eIF4E-binding protein 4E-BP1. When the activation of p53 is reversed within 16 h 4E-BP1 becomes rephosphorylated, the level of intact eIF4GI slowly increases and protein synthesis gradually recovers. The recovery of protein synthesis is partially blocked by rapamycin and wortmannin but not by the protein kinase inhibitors PD98059 and CGP74514A. Both rapamycin and wortmannin, but not PD98059 or CGP74514A, delay the reappearance of eIF4GI. In contrast, full-length 4E-BP1 rapidly becomes rephosphorylated and this process is partially inhibited by rapamycin, PD98059 and CGP74514A. Thus, activation of p53 results in the inhibition of distinct rapamycin- and wortmannin-sensitive pathways that target eIF4GI, and rapamycin-sensitive and -insensitive pathways that target 4E-BP1. Following inactivation of p53 the gradual recovery is determined largely by the kinetics of restoration of eIF4GI rather than by the rephosphorylation of full-length 4E-BP1. These findings suggest that the ability of cells to rephosphorylate 4E-BP1, resynthesise eIF4GI and restore the rate of protein synthesis after inactivation of p53 is an important aspect of recovery following the relief of physiological stress.

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Activation of the tumour suppressor protein p53 causes a rapid inhibition of protein synthesis which precedes the induction of a cell cycle block and subsequent apoptosis.^{1–3} The inhibition of translation is accompanied by early dephosphorylation and cleavage of the eukaryotic initiation factor (eIF)4E-binding protein 4E-BP1 as well as cleavage of polypeptide chain initiation factor eIF4GI and dephosphorylation of this protein at Ser¹¹⁰⁸.³ The cleaved products of 4E-BP1 and eIF4GI, which are generated by a caspase-independent mechanism, associate with eIF4E and there is a correspondingly decreased extent of interaction of eIF4E with full-length eIF4GI. These changes are likely to play an important role in impairing the initiation of protein synthesis.

In this study, we have examined whether the inhibition of protein synthesis can be reversed when a temperature-sensitive form of p53 is inactivated, prior to the irreversible commitment to cell death,² by return of the cells to the non-permissive temperature (38°C). We have also investigated the requirement for various signalling pathways that are known to regulate protein synthesis and have analysed the integrity and state of phosphorylation of eIF4GI and 4E-BP1 during the recovery phase. The results indicate a multifactorial mode of regulation of translation following the inactivation of p53 that is partially dependent on the activities of the protein kinase mammalian target of rapamycin (mTOR) and phosphatidylinositol-3-kinase (PI3k) but is independent of the

protein kinases Mek1/2 and cdc2. Furthermore, our findings suggest that the rate of recovery of protein synthesis is limited by the kinetics of reappearance of eIF4GI rather than by the extent of rephosphorylation of 4E-BP1 or the association of the latter with eIF4E.

Results

Characteristics of the recovery of protein synthesis following inactivation of p53. The activation of a temperature-sensitive form of p53 (Val¹³⁵) inhibits protein synthesis in MEL cells.¹ To determine if this inhibition is reversed when the cells are returned to the nonpermissive temperature for p53 activity (38°C), Val¹³⁵ cells and a control cell line with a nonactivatable form of p53 (Pro¹⁹⁰) were incubated at 32°C for 16 h and then returned to 38°C for various periods of time. Protein synthesis was then measured by [³⁵S]methionine incorporation. As shown in Figure 1a, although the control Pro¹⁹⁰ cells showed a small inhibition of protein synthesis when incubated at 32°C, upon return to 38°C they underwent a rapid recovery. In contrast, when the Val¹³⁵ cells were incubated at 32°C a much greater inhibition of protein synthesis was observed (Figure 1b). Moreover, protein synthesis did not immediately recover upon return of these cells to 38°C. After 8 and 24 h at 38°C

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Abbreviations: 4E-BP, eIF4E-binding protein; eIF, eukaryotic initiation factor; Erk, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; Mek, MAPK kinase; MEL, murine erythroleukaemia; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PI3k, phosphatidylinositol-3-kinase

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the rate had returned to only 49 and 77% of the control value, respectively (Figure 1b).

We have previously reported that although the mechanism by which protein synthesis is inhibited in cells with active p53 involves the dephosphorylation of the mTOR substrate 4E-BP1, this process is distinct from (and indeed additive with) the actions of the mTOR inhibitor rapamycin.³ It was therefore of interest to determine whether the recovery of protein synthesis is sensitive to rapamycin. When Val¹³⁵ cells were incubated at 32°C for 16h and then shifted back to the nonpermissive temperature in the presence or absence of 30 nM rapamycin, the mTOR inhibitor partially prevented the recovery of protein synthesis (Figure 2a). In cells allowed to recover for up to 24 h, rapamycin inhibited the restoration of protein synthesis by around 40–50%. Even in cells returned to 38°C for 48 h rapamycin still inhibited the recovery by approximately 44% (data not shown). The inhibition of protein synthesis in recovering cells is in contrast to the very small inhibition of translation (ca. 11–20%) that was observed when rapamycin was added to Pro¹⁹⁰ cells or to Val¹³⁵ cells that had been maintained at 38°C throughout (reference 3 and Figure 2b).

To examine the involvement of other signalling pathways in regulating the recovery of protein synthesis a number of other inhibitors were similarly added to the cells at the time of return to the nonpermissive temperature. As shown in Figure 2c, the PI3k inhibitor wortmannin also had a strong inhibitory effect on recovery. Like rapamycin, this agent was not strongly inhibitory towards cells prior to activation of p53 (Figure 2d). Nevertheless its presence resulted in a significant (ca. 50%) impairment of recovery in cells returned to 38°C for 24 h (Figure 2c). It is well established that mTOR is regulated in a PI3k-dependent manner, via the action of the protein kinase Akt.⁴ It was therefore possible that wortmannin and rapamycin were both acting on the same pathway to cause impairment of mTOR activity. However, the addition of both inhibitors had a significant additive effect on protein synthesis. This was observed both in Val¹³⁵ cells incubated at 32°C (where the inhibitors further increased the inhibition due to p53 activation – Figure 3a) and in cells recovering after 24 h at 38°C (Figure 3b). These data suggest that at least two distinct pathways, sensitive to wortmannin and rapamycin respectively, are required for the reactivation of protein synthesis during the recovery from p53 inhibition.

The Mek1/2 kinase inhibitor PD98059 had only a small effect on the recovering cells (Figure 4a). This was not specific to p53-mediated regulation since the PD98059 was also somewhat inhibitory towards control cells at 38°C (Figure 4b). On the other hand, the p38 MAP kinase inhibitor SB203580 was very inhibitory to protein synthesis under all conditions (Figure 4a and b). Both inhibitors were active against their respective target enzymes (Figure 4c). Our results suggest that these inhibitors, especially SB203580, block pathways that are constitutively required for protein synthesis, independently of whether p53 is activated. In contrast, the mTOR and PI3k inhibitors block pathways that are only necessary for the recovery from p53-induced inhibition, and the compounds have very little effect when p53 is inactive. The cdc2 kinase inhibitor CGP74514A had very little effect on the recovery of protein synthesis from inhibition by p53, in the presence or

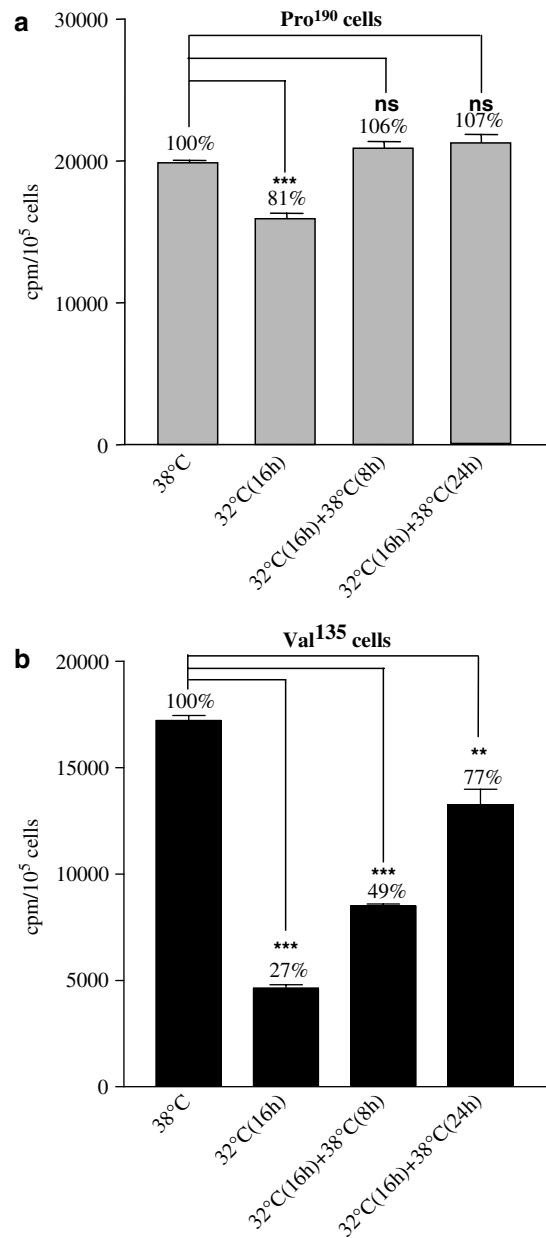


Figure 1 Inhibition of p53 activity results in slow recovery of the rate of protein synthesis. Pro¹⁹⁰ cells (a) and Val¹³⁵ cells (b) that had been cultured at 38°C were shifted to 32°C for 16 h and then shifted back to 38°C for the periods of time indicated. Rates of protein synthesis were measured during the last hour of incubation by pulse-labelling the cells with [³⁵S]methionine (10 μCi/ml) as described in Materials and Methods. The data are expressed as radioactivity incorporated per 10⁵ cells ± S.E.M. Relative rates of incorporation, expressed as % of the control rate for each cell line (cells maintained at 38°C throughout), are shown above each bar. Statistical significances of differences between the values shown were calculated by paired t-test; ns indicates not significant ($P=0.05$ or greater), ** indicates $P<0.001$, *** indicates $P<0.0001$

absence of rapamycin (Figure 4d). This compound was active under the conditions used since it caused the dephosphorylation of cdc2 at Thr¹⁶¹ (Figure 4e). Thus, in spite of the ability of p53 to inhibit cdc2,⁵ activity of the latter kinase does not seem to be required for the restoration of translation.

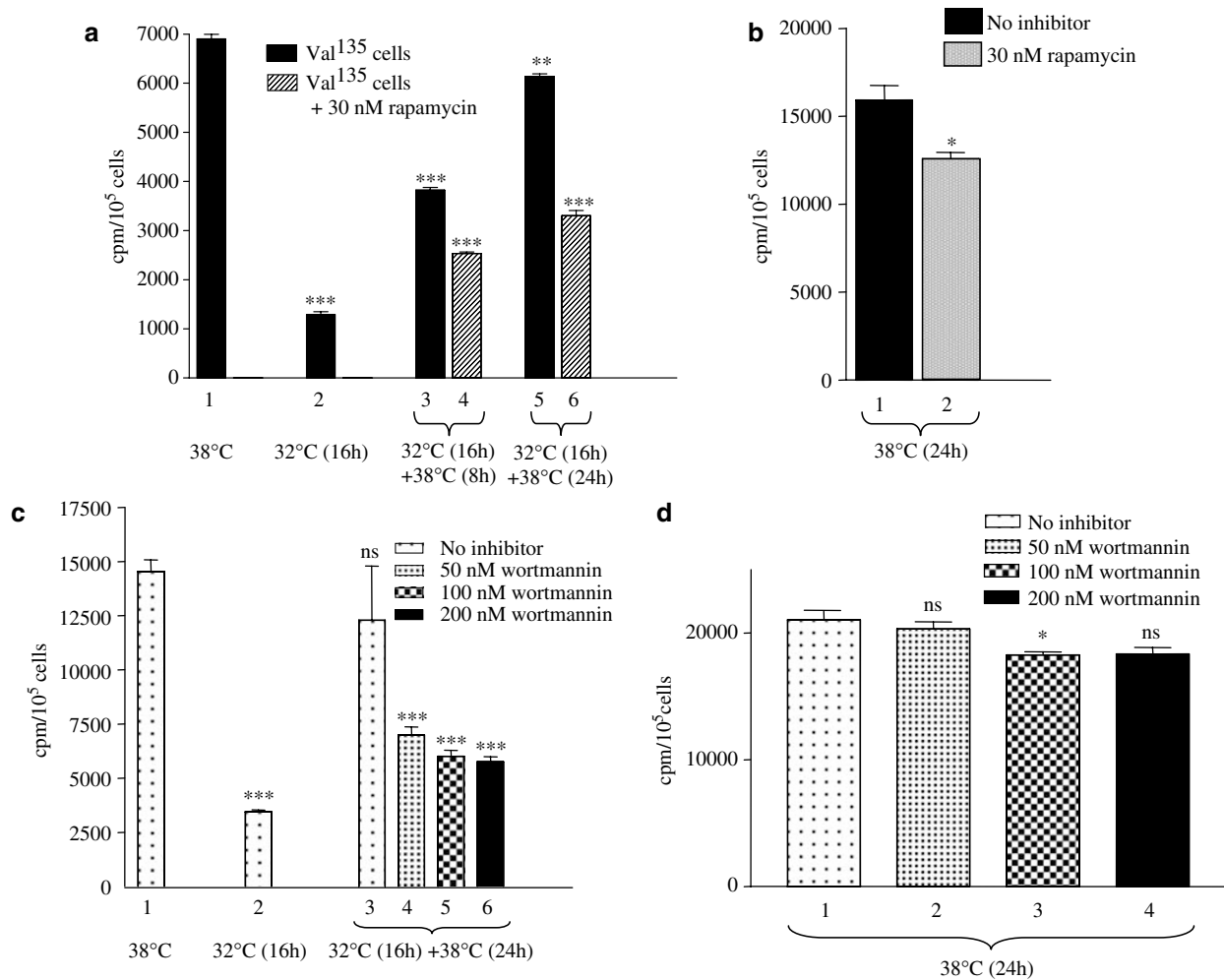


Figure 2 Inhibition by rapamycin and wortmannin of the recovery of protein synthesis following inactivation of p53. (a) Val¹³⁵ cells that had been incubated at 38°C were shifted to 32°C for 16 h. The cells were then returned to 38°C for the times shown, in the absence (black bars) or presence (shaded bars) of 30 nM rapamycin. (b) Val¹³⁵ cells that had been maintained at 38°C throughout were incubated with or without rapamycin for 24 h. (c) Val¹³⁵ cells that had been incubated at 38°C were shifted to 32°C for 16 h and then returned to 38°C for 24 h in the presence of the indicated concentrations of wortmannin. (d) Control cells that had been maintained at 38°C throughout were incubated with the same concentrations of wortmannin for 24 h. Rates of protein synthesis were measured as described in Figure 1. Statistical significances of differences between values were calculated by paired t-test. For panel a, ** indicates $P < 0.001$ and *** indicates $P < 0.0001$ for the differences between bars 1 versus 2, 1 versus 3, 1 versus 5, 3 versus 4 and 5 versus 6; for panel b, * indicates $P < 0.01$ for the difference between bars 1 and 2; for panel c, ns indicates not significant and *** indicates $P < 0.0001$ for the differences between bars 1 versus 2, 1 versus 3, 3 versus 4, 3 versus 5 and 3 versus 6; for panel d, ns indicates not significant and * indicates $P = 0.01$ for the differences between bars 1 versus 2, 1 versus 3 and 1 versus 4. Note that, at the concentrations employed here, wortmannin has only a minimal direct effect on the activity of mTOR^{30,31}

Effects of rapamycin and wortmannin on the level of initiation factor eIF4GI during the recovery of protein synthesis. We have investigated the kinetics of reversal of the various changes in the initiation machinery associated with inhibition of translation by p53.³ To examine the rate of reappearance of full-length initiation factor eIF4GI and the disappearance of the previously described cleaved form of the factor,³ lysates from Val¹³⁵ cells were analysed by SDS gel electrophoresis and immunoblotting. Two different anti-eIF4GI antibodies were used in this analysis, one of which recognizes only the full-length (uncleaved) factor whereas the other also detects a ca. 80 kDa cleavage product derived from the central region of eIF4GI. As shown in Figure 5a (lanes 2, 3 and 5), restoration of full-length eIF4GI did not

occur within 8 h after the shift back to 38°C (indeed the signal further decreased), and recovery was less than complete even at 24 h. In contrast the majority of the p53-induced cleavage product of eIF4GI, which was detectable after the cells had been incubated at 16 h at 32°C, disappeared within 8 h after p53 was inactivated (Figure 5b, lanes 2, 3 and 5). In view of the low level of eIF4GI in cells with active p53 the slow reappearance of the factor during the recovery is likely to be mainly determined by the kinetics of resynthesis. Comparison of the results in Figure 5 with those in Figure 1 suggests that the extent of reappearance of full-length eIF4GI may determine the rate of recovery of protein synthesis when p53 is inactivated. However the small residual amount of the intact factor (Figure 5a, lane 3) may

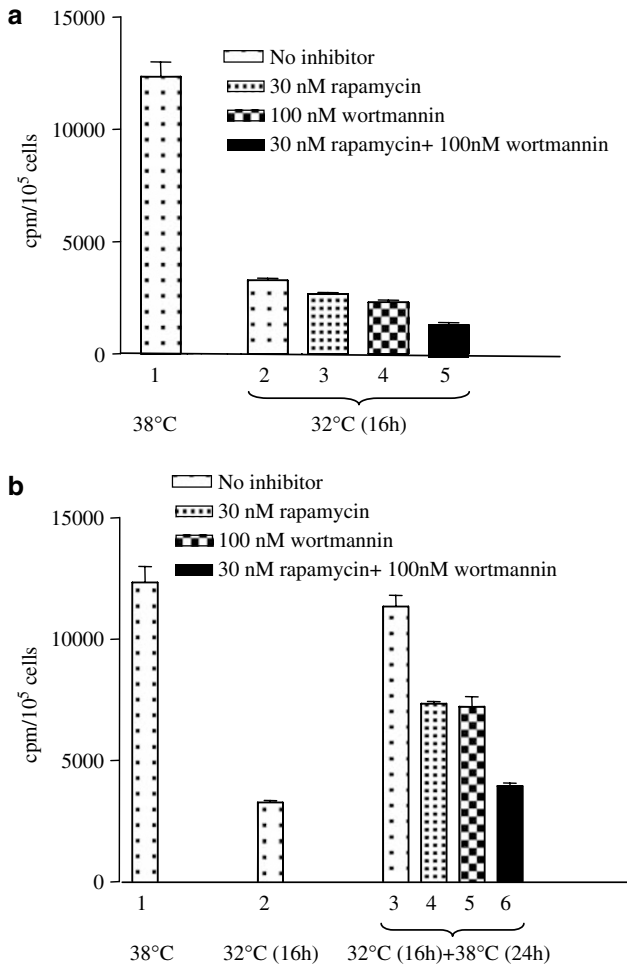


Figure 3 Rapamycin and wortmannin have additive inhibitory effects on the recovery of protein synthesis following inactivation of p53. **(a)** Val¹³⁵ cells that had been incubated at 38°C were shifted to 32°C for 16 h in the absence or presence of rapamycin (30 nM) and/or wortmannin (100 nM) as indicated. **(b)** Cells that had been incubated at 32°C for 16 h in the absence of inhibitors were returned to 38°C for 24 h in the absence or presence of rapamycin and/or wortmannin. In each case rates of protein synthesis were measured as described in Figure 1. The rates of protein synthesis at 38°C in the absence of inhibitors are shown for comparison. Statistical significances of differences between values were calculated by paired t test. For panel **a**, $P < 0.01$ for the difference between bar 1 versus 2, $P < 0.001$ for the difference between bar 1 versus 3, $P < 0.0001$ for the differences between bar 1 versus bars 4 and 5, $P < 0.01$ for the differences between bar 2 versus bars 3 and 5, $P = 0.03$ for the difference between bar 2 versus 4, $P < 0.01$ for the difference between bar 3 versus 5, $P < 0.001$ for the difference between bar 4 versus 5; for panel **b**, $P < 0.01$ for the difference between bar 1 versus 2, there is no significant difference between bars 1 versus 3, $P < 0.001$ for the difference between bar 3 versus 4, $P = 0.001$ for the difference between bar 3 versus 5, $P < 0.0001$ for the difference between bar 3 versus 6, and $P < 0.001$ for the differences between bar 4 versus 6 and bar 5 versus 6

be sufficient to sustain a basal rate of translation and permit some recovery at 8 h, especially since the 80 kDa cleavage product of eIF4G1 has largely disappeared by this time (Figure 5b, lane 3).

As rapamycin partially blocks the restoration of protein synthesis following the reversal of p53 activity we examined the effects of this inhibitor on eIF4G1 levels. Addition of

rapamycin when the cells were shifted back to the non-permissive temperature strongly inhibited the reappearance of full-length eIF4G1 (Figure 5a, lanes 4 and 6). In contrast the mTOR inhibitor did not prevent loss of the eIF4G1 cleavage product (Figure 5b, lanes 4 and 6). These results suggest that eIF4G1 synthesis in recovering cells is particularly sensitive to inhibition of mTOR activity. However, rapamycin had little effect on the level of full-length eIF4G1 in control cells at 38°C (Figure 5c). This is consistent with the very small effect of the inhibitor on protein synthesis under these conditions (Figure 2b) and may be a reflection of a relatively high stability of eIF4G1 in the absence of p53 activation.

Wortmannin caused a similar delay in the reappearance of eIF4G1 (Figure 6a) but, as with rapamycin, did not prevent the disappearance of the cleavage product (Figure 6b). Again these data are consistent with the rate of recovery of protein synthesis being limited by the absence of full-length eIF4G1. Wortmannin had little effect on the level of eIF4G1 in control cells at 38°C (Figure 6c). PD98059, SB203580 and CGP74514A all had only minor effects on the reappearance of full-length eIF4G1 during the recovery period (data not shown). These results argue against a major role for pathways dependent on Mek1/2 kinase, p38 MAP kinase or cdc2 kinase activities in the regulation of eIF4G1 levels.

Figure 6a (middle panel) also shows that when the Val¹³⁵ cells were shifted to 32°C initiation factor eIF4G1 was dephosphorylated at Ser¹¹⁰⁸. Complete rephosphorylation of this site took place within 24 h when the cells were returned to the nonpermissive temperature, but this was prevented in the presence of either rapamycin or wortmannin.

Regulation of 4E-BP1 and its association with eIF4E during the recovery of protein synthesis.

Activation of p53 causes both the dephosphorylation of 4E-BP1 and the cleavage of this protein to generate two truncated forms. Both the hypophosphorylated full-length and the larger cleaved form of the protein bind to eIF4E.³ To examine the integrity and state of phosphorylation of 4E-BP1 during the recovery of protein synthesis, cell extracts were separated on SDS gels and immunoblotted with antibodies to total and phosphorylated 4E-BP1. Figure 7a (top panel) shows that the truncated forms of 4E-BP1 partially disappeared within 8 h after the return of the cells to 38°C and were completely lost by 24 h. Extensive dephosphorylation at Ser⁶⁴, and partial dephosphorylation at Thr⁶⁹ and Thr^{36/45}, occurred at 32°C but reincubation at 38°C resulted in complete rephosphorylation of the full-length protein at all these sites within 8 h (Figure 7a and Supplementary Figure 1). This indicates rapid reversal of the activities of 4E-BP1-specific protein kinases and/or phosphatases that are regulated by p53. In contrast, even though the truncated forms of 4E-BP1 were still detectable at 8 h, no re-phosphorylation of these proteins took place (Figure 7a, lane 3). This indicates that these forms of 4E-BP1 remain irreversibly hypophosphorylated at Ser⁶⁴, Thr⁶⁹ and Thr^{36/45} under conditions where the full-length protein does become re-phosphorylated. This may delay the release of eIF4E from the inhibitory effect of the larger truncated form of 4E-BP1, to which the initiation factor is bound (see below).

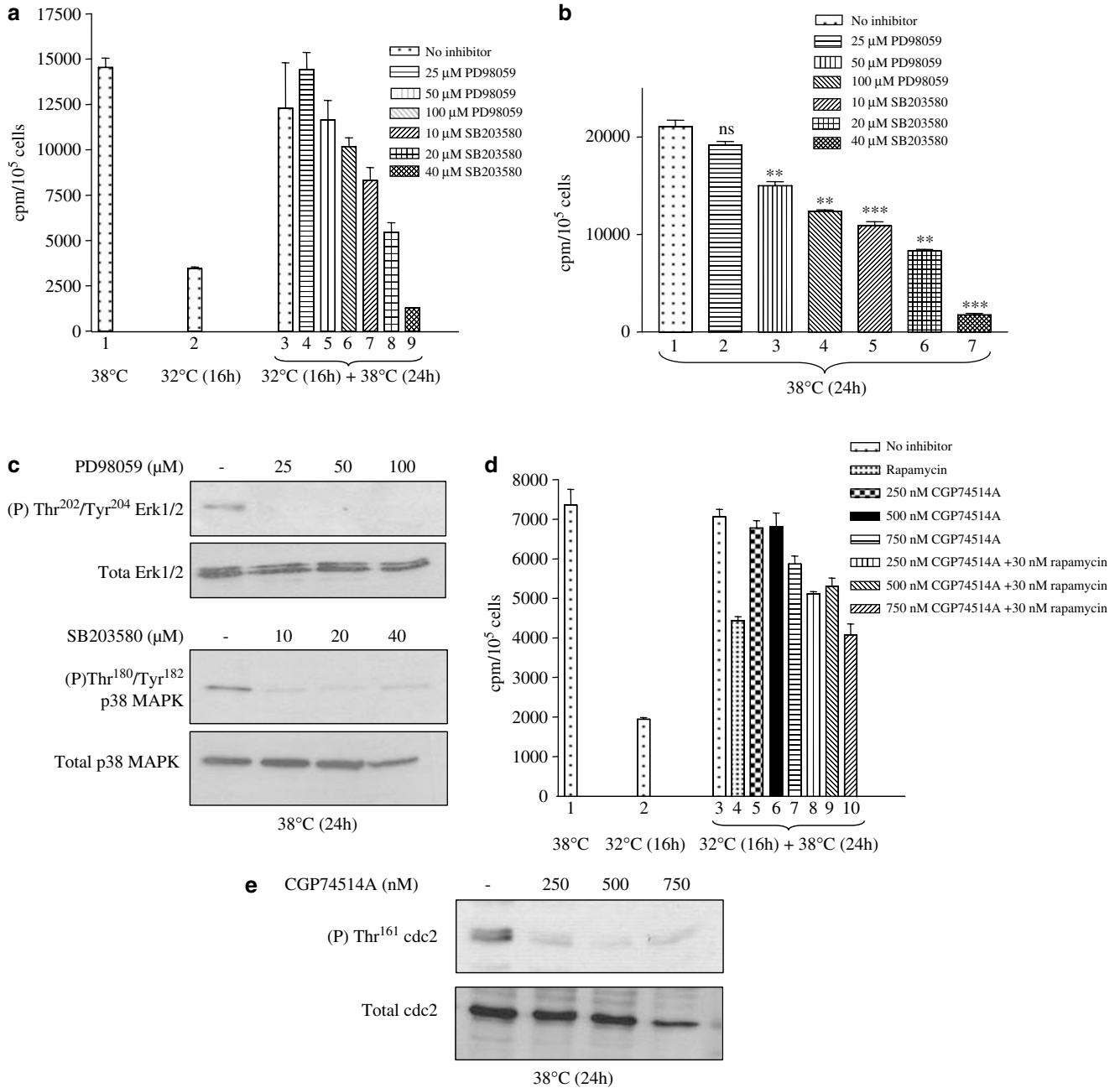


Figure 4 Effects of inhibitors of Mek1/2 kinases, p38 MAP kinase and cdc2 kinase on the recovery of protein synthesis after inactivation of p53. **(a)** Val¹³⁵ cells that had been growing at 38°C were shifted to 32°C for 16 h. The cells were then returned to 38°C for 24 h in the absence or presence of PD98059 or SB203580 at the concentrations indicated. **(b)** Val¹³⁵ cells were maintained at 38°C and incubated for 24 h in the absence or presence of the same inhibitor concentrations as in **(a)**. **(c)** Extracts prepared from cells incubated with the indicated concentrations of PD98059 or SB203580 were analysed by SDS gel electrophoresis and immunoblotting for phosphorylated Erk1/2 (Thr²⁰²/Tyr²⁰⁴), total Erk1/2, phosphorylated p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) and total p38 MAPK as indicated. **(d)** Val¹³⁵ cells were treated as in **(a)**, except that CGP74514A was added at the concentrations indicated, in the absence or presence of rapamycin (30 nM). **(e)** Extracts prepared from cells incubated with the indicated concentrations of CGP74514A were analysed by SDS gel electrophoresis and immunoblotting for phosphorylated cdc2 (Thr¹⁶¹) and total cdc2 as indicated. Rates of protein synthesis (panels **a**, **b** and **d**) were measured as described in Figure 1. Statistical significances of differences between values were calculated by paired t test. For panel **a**, $P < 0.001$ for the difference between bar 1 versus 2, there is no significant difference between bar 1 versus 3 or between bar 3 versus bars 4 and 5, $P < 0.01$ for the difference between bar 3 versus 6, $P = 0.02$ for the difference between bar 3 versus 7, $P < 0.001$ for the difference between bar 3 versus 8, and $P < 0.0001$ for the difference between bar 3 versus 9; for panel **b**, ns indicates not significant, ** indicates $P < 0.01$ and *** indicates $P < 0.0001$ for the differences between bar 1 versus the bars shown; for panel **d**, $P < 0.001$ for the difference between bar 1 versus 2, there is no significant difference between bar 1 versus 3 or between bar 3 versus bars 5 and 6, $P < 0.02$ for the difference between bar 3 versus 7, $P < 0.01$ for the difference between bar 3 versus 8, $P < 0.001$ for the differences between bar 3 versus bars 4 and 10, and $P < 0.0001$ for the difference between bar 3 versus 9

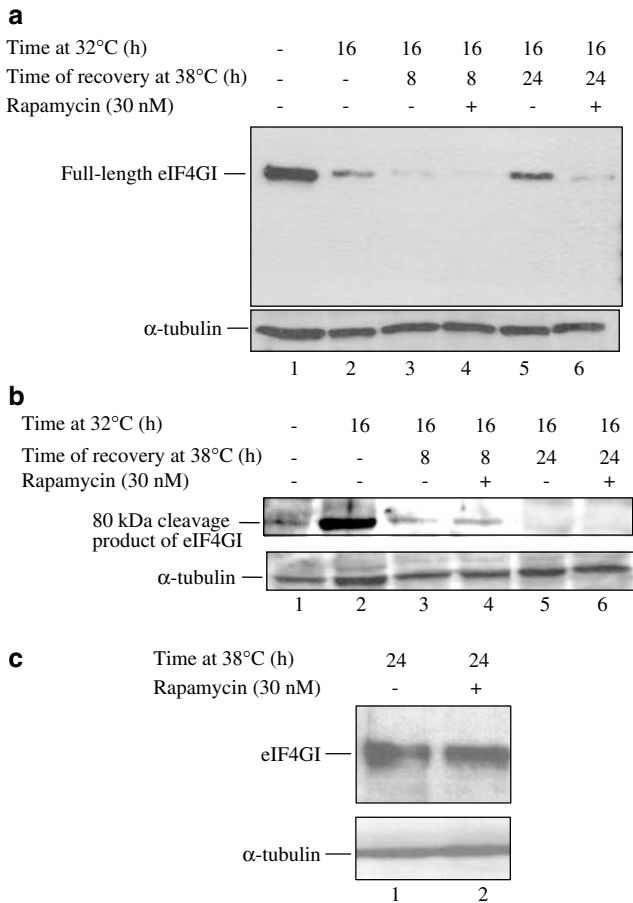


Figure 5 Inhibition by rapamycin of the reappearance of full-length initiation factor eIF4GI during the recovery of protein synthesis from inhibition by p53. Val¹³⁵ cells that had been grown at 38°C (lane 1) were shifted to 32°C for 16 h (lane 2) and then returned to 38°C for 8 h (lanes 3 and 4) or 24 h (lanes 5 and 6). Where indicated (lanes 4 and 6), rapamycin (30 nM) was added when the cells were returned to 38°C. Cell extracts were prepared as described in Materials and Methods, separated on SDS polyacrylamide gels and immunoblotted for (a) full-length eIF4GI or (b) the ca. 80 kDa cleavage product of eIF4GI. The blots were then re-probed for α -tubulin as a loading control. The positions of the relevant bands are indicated. (c) Cells that had been maintained at 38°C throughout were incubated with or without rapamycin for 24 h and extracts were blotted for full-length eIF4GI and α -tubulin as above

We also investigated the effects of rapamycin and other signal transduction inhibitors on the rephosphorylation of 4E-BP1. Figure 7a and Supplementary Figure 1 show that upon return of the Val¹³⁵ cells to the nonpermissive temperature full-length 4E-BP1 became partially rephosphorylated at Ser⁶⁴, Thr⁶⁹ and Thr^{36/45} even in the presence of rapamycin. These data suggest that although the rephosphorylation of these sites probably requires an active mTOR-raptor complex, other protein kinases, or possibly a rapamycin-resistant form of mTOR, are also involved. Similar analyses of 4E-BP1 rephosphorylation were performed on cells restored to 38°C in the presence of wortmannin. However in this case the data were difficult to interpret because of a major loss of total 4E-BP1 protein (data not shown).

PD98059, SB203580 and CGP74514A all inhibited the rephosphorylation of full-length 4E-BP1 to some extent

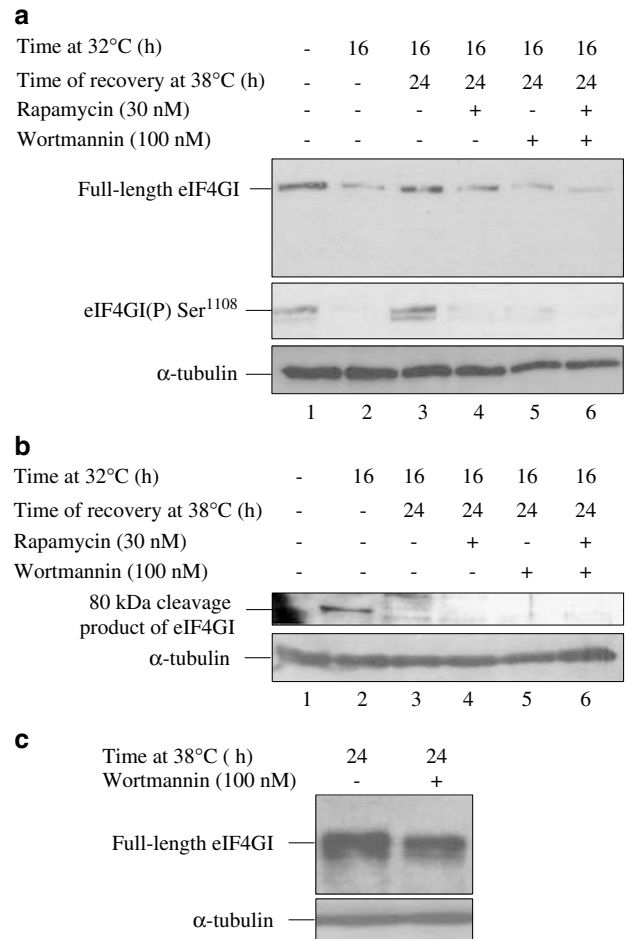


Figure 6 Inhibition by rapamycin and wortmannin of the reappearance and rephosphorylation of full-length eIF4GI during the recovery phase of protein synthesis. Val¹³⁵ cells that had been grown at 38°C were shifted to 32°C for 16 h. The cells were then returned to 38°C and incubated in the absence or presence of rapamycin (30 nM) and/or wortmannin (100 nM) for 24 h. Cell extracts were prepared as described in Materials and Methods, separated on SDS polyacrylamide gels and immunoblotted for (a) total full-length eIF4GI and eIF4GI phosphorylated at Ser¹¹⁰⁸ and (b) the ca. 80 kDa cleavage product of eIF4GI. The blots were then re-probed for α -tubulin as a loading control. The positions of the relevant bands are indicated. (c) Val¹³⁵ cells that had been maintained at 38°C throughout were incubated with or without wortmannin for 24 h and extracts were blotted for total full-length eIF4GI and α -tubulin as above

(especially at Ser⁶⁴) when the Val¹³⁵ cells were restored to 38°C (Figure 7b). The effects of PD98059 and CGP74514A are consistent with previous observations that 4E-BP1 can be phosphorylated by the Erks⁶ and cdc2 kinase.⁷ As indicated above, however, neither Mek/Erk inhibition nor cdc2 inhibition impaired the recovery of protein synthesis (Figure 4). These results lead us to conclude that the availability of eIF4E (i.e. in a form not complexed with 4E-BP1) must be adequate to meet the translational demand 24 h after the shift back to 38°C, even when the activities of the Erk and cdc2 kinases are compromised.

Cell extracts were also analysed for the extent of association of 4E-BP1 with eIF4E, using m⁷GTP-Sepharose affinity chromatography. Figure 8a shows that, surprisingly, there was relatively little change in the binding of full-length 4E-BP1

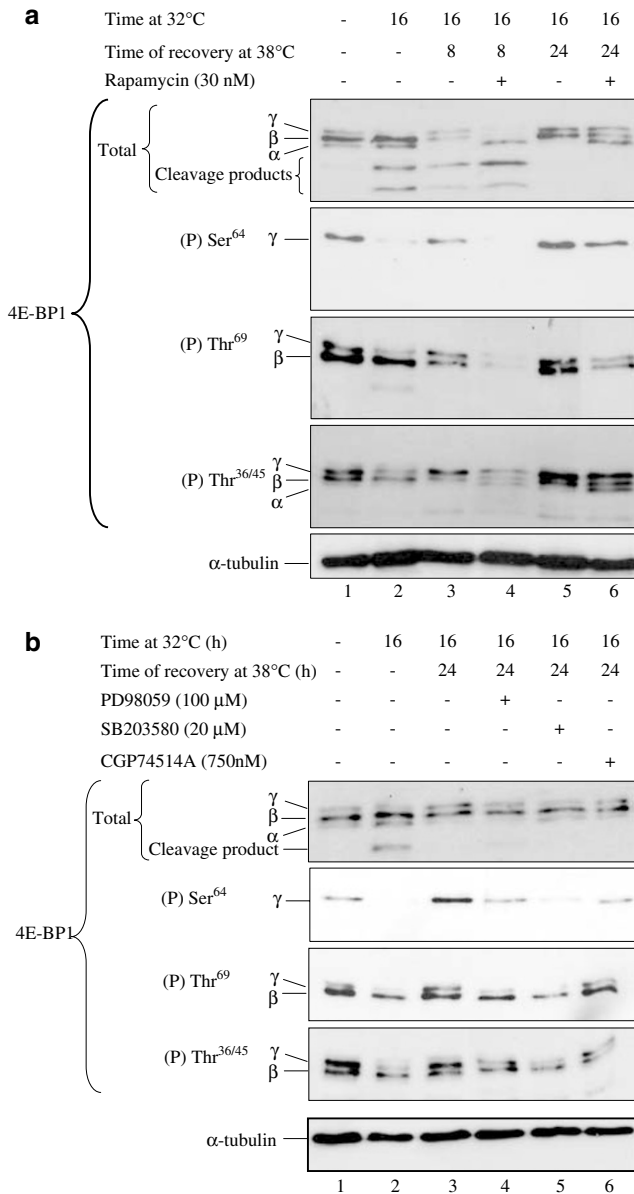


Figure 7 Changes in the integrity and phosphorylation of 4E-BP1 during the recovery phase of protein synthesis and effects of inhibitors of signal transduction pathways. (a) Val¹³⁵ cells that had been grown at 38°C were shifted to 32°C for 16 h and then returned to 38°C for 8 h or 24 h, in the absence or presence of rapamycin (30 nM). Cell extracts were prepared, separated on SDS polyacrylamide gels and immunoblotted for total 4E-BP1 and 4E-BP1 phosphorylated on Ser⁶⁴, Thr⁶⁹ and Thr^{36/45}. The positions of the differentially phosphorylated α , β and γ forms of full-length 4E-BP1 and of two truncated forms (cleavage products) are indicated. The blots were re-probed for α -tubulin as a loading control. (b) Val¹³⁵ cells that had been grown at 38°C were shifted to 32°C for 16 h and then returned to 38°C for 24 h, in the absence or presence of PD98058 (100 μ M), SB203580 (20 μ M) or CGP74514A (750 nM) as indicated. Cell extracts were prepared and immunoblotted as in (a). The positions of the differentially phosphorylated α , β and γ forms of full-length 4E-BP1 and of the larger truncated form (cleavage product) are indicated

to eIF4E when the cells were shifted between 38 and 32°C and *vice versa*. It was notable that the larger truncated form of 4E-BP1 bound strongly to eIF4E after activation of p53, and remained associated with the factor at the 8 h recovery time

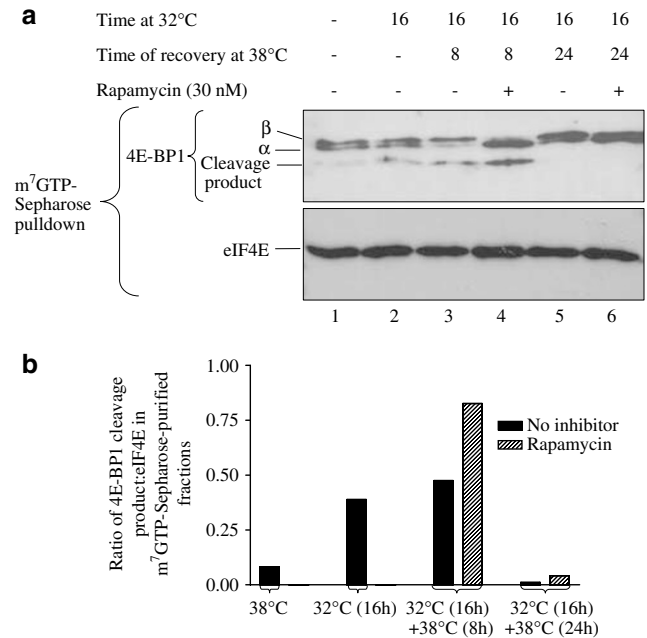


Figure 8 Binding of full-length and truncated 4E-BP1 to eIF4E during the recovery phase of protein synthesis. Val¹³⁵ cells that had been grown at 38°C were shifted to 32°C for 16 h and then returned to 38°C for 8 h or 24 h, in the absence or presence of rapamycin (30 nM). Cell extracts were prepared and m⁷GTP-Sepharose affinity chromatography performed as described in Materials and Methods. (a) Affinity-purified fractions obtained from equal amounts of cell extract were separated on SDS polyacrylamide gels and immunoblotted for total 4E-BP1 and eIF4E as indicated. (b) The band intensities of the bound cleavage product of 4E-BP1 and of eIF4E in the m⁷GTP-Sepharose-purified fractions were quantified by densitometry and are shown as ratios

point (Figure 8a, lanes 2 and 3 and Figure 8b). However at 24 h no truncated 4E-BP1 remained associated with eIF4E (Figure 8a, lanes 5 and 6). The smaller truncated form of 4E-BP1 did not bind to eIF4E under any conditions. Addition of rapamycin at the time of the shift back to 38°C increased the extent of binding of the larger truncated form to eIF4E at 8 h (Figure 8a, lanes 3 and 4, and Figure 8b), but the inhibitor did not prevent the loss of this binding at 24 h (Figure 8a, lanes 5 and 6). Although rapamycin delayed the rephosphorylation of full-length 4E-BP1, and increased the binding of this protein to eIF4E at 8 h, it had no effect on the binding of 4E-BP1 to eIF4E at 24 h (Figure 8a, compare lanes 5 and 6). The latter result suggests that the ability of rapamycin to inhibit the recovery of protein synthesis at 24 h (and later time points) cannot be ascribed to regulation of the availability of eIF4E by either full-length or truncated 4E-BP1.

Discussion

Signalling pathways required for recovery of protein synthesis from p53-induced inhibition. The gradual recovery of protein synthesis that occurs when p53 is inactivated is consistent with our previous conclusion that MEL cells are not irreversibly committed to apoptosis or loss of viability by activation of p53 for up to 16 h.² This indicates that the signal transduction pathways and mechanisms for

the restoration of the activity of the translational apparatus are not irreversibly compromised within this time frame.

We previously concluded that inhibition of mTOR activity plays only a minor role in mediating the inhibitory effects of p53.³ However, exposure of the cells to rapamycin upon returning them to the nonpermissive temperature partially inhibits the recovery of protein synthesis, to an extent which is much greater than the inhibition observed in cells incubated throughout at 38°C. These data suggest that the rapamycin-sensitive mTOR-raptor complex plays a more significant role in cells recovering from the effects of this physiological stress than in control cells. One possible reason is that mTOR may selectively regulate the translation of mRNAs encoding components of the translation machinery which are depleted as a result of p53 activation.⁸ We have shown here that rapamycin strongly impairs the restoration of the level of eIF4GI following inactivation of p53 (Figures 5 and 6) and new synthesis of this factor is likely to be required for the reacquisition of full translational capacity. The fact that rapamycin does not fully prevent the recovery of protein synthesis suggests, in addition, the existence of one or more pathways for translational regulation by p53 that are independent of rapamycin-sensitive mTOR. This is in agreement with the distinct and additive effects of p53 activation and rapamycin treatment on 4E-BP1 dephosphorylation, eIF4F complex formation and protein synthesis rates in MEL cells.³ The low residual level of eIF4GI present in cells at 8 h of recovery (and at later times also in rapamycin-treated cells) may be sufficient to sustain a basal rate of protein synthesis and allow a small degree of recovery but this level is likely to be rate-limiting for translation at later time points.

Wortmannin also partially prevents the recovery of protein synthesis following the inactivation of p53, and this inhibition is likewise associated with the failure of eIF4GI to be restored to control levels (Figure 6). The mechanism whereby PI3k activity influences eIF4GI synthesis is unclear. It may act via regulation of downstream Akt, which in turn acts on mTOR,⁹ but the involvement of one or more additional pathways is also likely. We favour the latter interpretation because: (i) we have not seen any evidence for inhibition of Akt activity in cells with activated p53¹; (ii) there is no decrease in the phosphorylation of Akt on Ser⁴⁷³ (a site associated with activation of this protein kinase) following p53 activation, although there is hyperphosphorylation of this site during the recovery phase (Supplementary Figure 2); and (iii) the effects of wortmannin and rapamycin on protein synthesis during recovery are additive, suggesting an mTOR-independent mode of action of the PI3k inhibitor. Interestingly, mTOR-independent but PI3k-dependent phosphorylation of 4E-BP1 has previously been reported.¹⁰ Whatever the mechanism it is important to note that, although the PI3k/Akt pathway provides an important survival signal for MEL cells,¹¹ neither rapamycin nor wortmannin significantly affects the level of eIF4GI in exponentially growing control cells (Figures 5c and 6c).

Mek1/2 kinase activity is not required for restoration of translation when p53 is inactivated and we have observed increases rather than decreases in the levels of activating phosphorylations of both Mek1/2 and Erk1/2 kinases following the shift of cells to 32°C (Supplementary Figure 3). This is consistent with the conclusion that Mek and Erk activity is

required for p53-dependent apoptosis in this system.¹² The ability of PD98059 to inhibit the rephosphorylation of full-length 4E-BP1 but not block the recovery of protein synthesis at 38°C suggests that the extent of phosphorylation of 4E-BP1 (particularly at Ser⁶⁴) is not critical in controlling the availability of eIF4E for translation under these conditions.

Although there are indications that cdc2 activity is inhibited by the activation of p53 in MEL cells,³ CGP74514A was ineffective in blocking the recovery of protein synthesis (Figure 4d). Nevertheless, there was some inhibition of the rephosphorylation of Ser⁶⁴ in cells returned to 38°C in the presence of the cdc2 inhibitor (Figure 7b), again suggesting that the impairment of Ser⁶⁴ phosphorylation is not critical to the recovery process.

We have not been able to assess the specific importance of the p38 MAP kinase pathway in the response of MEL cells to p53 because SB203580 was also strongly inhibitory for protein synthesis in control cells (Figure 4b). However, p53 activation caused only a very small decrease in the activity of p38 MAP kinase (as judged by the state of phosphorylation of this protein on Thr¹⁸⁰/Tyr¹⁸²) (Supplementary Figure 4).

Relative roles of eIF4GI and 4E-BP1 modifications in regulation of translation by p53.

Our data suggest that, whereas during the first 8 h of recovery the binding of the larger truncated form of 4E-BP1 to eIF4E may limit the rate of protein synthesis, at later times (when this protein has disappeared) the submaximal rates of protein synthesis are more likely to be due to the lack of full-length eIF4GI. This conclusion is confirmed by the effects of rapamycin and wortmannin, which block the restoration of full-length eIF4GI. In addition, the fact that the eIF4GI cleavage product disappears well in advance of the recovery of translation indicates that the slow restoration of protein synthesis is not attributable to the effects of this protein.

The mechanisms by which the restoration of eIF4GI is inhibited by rapamycin or wortmannin remain to be established. There are several precedents for the sensitivity of translation of specific mRNAs to inhibitors of the PI3k/mTOR pathway and microarray and other studies have revealed a large number of mRNAs that are translationally repressed by rapamycin.¹³ These include mRNAs encoding ribosomal proteins, as well as polypeptide chain initiation factors eIF4A, eIF5A and eIF2B ϵ .^{13,14} In the case of eIF4GI the mRNA for this large protein can be translated by internal initiation under certain conditions and this might be expected to render the synthesis of eIF4GI insensitive to rapamycin. Curiously, however, the internal ribosome entry site-mediated translation of eIF4GI still demonstrates cap-dependence.¹⁵ It has also been suggested that rapamycin causes decreased transcription of the eIF4G gene and, additionally, can enhance the rate of proteolysis of the initiation factor.^{16,17} We cannot formally rule out the possibility that rapamycin regulates the expression of eIF4GI by such mechanisms. Nevertheless, in the absence of p53 activation neither rapamycin nor wortmannin has any effect on the level of the protein (Figures 5c and 6c).

Upon return of the cells to 38°C full-length 4E-BP1 becomes rapidly rephosphorylated at all sites examined (Figure 7 and Supplementary Figure 1). This occurs well before protein synthesis is fully restored suggesting that, at later time points,

the state of phosphorylation of 4E-BP1 may be less important than the level or integrity of eIF4G1 in determining the overall rate of translation. Even in the presence of rapamycin partial rephosphorylation of 4E-BP1 occurred within 24 h, providing evidence that one or more kinases other than rapamycin-sensitive mTOR must be involved in regulating the phosphorylation of the translational repressor. It is possible that different protein kinases may phosphorylate different sites on 4E-BP1,¹⁸ and/or that both rapamycin-sensitive and -insensitive forms of mTOR¹⁹ are involved.

In spite of the changes in the phosphorylation of 4E-BP1 there is relatively little difference in the amounts of full-length 4E-BP1 bound to eIF4E in cells shifted from 38 to 32°C and back again (Figure 8a). This argues against a major role for 4E-BP1 phosphorylation in regulating protein synthesis in MEL cells following changes in p53 activity. Consistent with this, PD98059 and CGP74514A inhibit the rephosphorylation of 4E-BP1 (especially at Ser⁶⁴) without impairing the recovery of protein synthesis. However, the larger cleavage product of 4E-BP1 present in cells with active p53 (reference 3 and Figure 7) binds strongly to eIF4E (Figure 8a) and may limit the availability of the latter during the early phase of recovery. Addition of rapamycin at the time of shifting the cells back to 38°C increases the binding of this protein to eIF4E at 8 h (Figure 8a). However, the truncated 4E-BP1 is lost after 24 h of recovery and cannot therefore be responsible for the continuing depressed rate of translation at this time.

Significance for p53-mediated cellular responses to physiological stresses. The activation of p53 by cellular stresses such as DNA damage is well known to induce cell growth arrest and/or apoptosis, and the shut-down of protein synthesis can be viewed as a component of both these effects. However, even when the eventual outcome is cell death there is an initial period during which the effects of p53 are reversible, at least in transformed cells.^{20,21} MEL cells do not become committed to apoptosis until at least 16 h after the activation of the tumour suppressor protein.² Moreover, in another system utilizing inducible p53, where protein synthesis is strongly impaired following induction, there is no obvious apoptosis for up to 4 days.²² The ability to rephosphorylate 4E-BP1, resynthesise eIF4G1 and restore the rate of protein synthesis after inactivation of p53 is likely to be an important factor in cellular recovery.

Interestingly, a recent report²³ has shown that over-expression of eIF4E can inhibit p53-mediated transcription and the initiation of apoptosis. This may be explained by the promotion of the synthesis of anti-apoptotic proteins such as p21 and survivin, the translation of which is particularly dependent on mTOR activity.^{13,24} Our results, in conjunction with these data, suggest that an amplification loop may exist whereby the activation of p53 inhibits eIF4E function (via dephosphorylation of 4E-BP1 and the loss of eIF4G) and this in turn allows p53 to remain active for longer. However, in tumours where eIF4E is over-expressed,^{25–27} or where the PI3k/Akt/mTOR pathway is hyperactive,²⁸ such positive feedback may be inhibited and the cells would thus be more resistant to the effects of p53 activation. This could explain the anti-apoptotic and transforming effects of high levels of eIF4E.

Materials and Methods

Cell Culture Conditions. The Val¹³⁵ and Pro¹⁹⁰ MEL cell lines were obtained from Dr S Benchimol²⁹ and were grown in stationary suspension culture in DMEM medium supplemented with glutamine (300 mg/L) and 10% (v/v) foetal bovine serum in a 5% CO₂ atmosphere at 38°C. Under these conditions the expressed p53 was inactive and the cells grew exponentially with a doubling time of 12 h. Cultures were maintained at densities between 2 and 10 × 10⁵ cells per ml. Continued expression of p53 was assured by weekly selection of the cells in G418 (200 µg/ml). For activation of p53 in the Val¹³⁵ cells the cultures were transferred to 32°C for the times indicated. The control Pro¹⁹⁰ cells (in which the p53 is inactive at both temperatures) were treated similarly. The inhibitors rapamycin, wortmannin, PD98059, SB203580 and CGP74514A (Calbiochem) were added to the cells at the concentrations and for the times described in the figure legends.

Measurement of protein synthesis rates. Cells were counted in a haemocytometer and overall rates of protein synthesis were then measured by pulse labelling for 1 h with 6–10 µCi/ml of [³⁵S]methionine (in the presence of the normal level of methionine in the cell culture medium). After the incubation 300 µl of cells were added to 5 ml of phosphate-buffered saline (PBS) and centrifuged at 1000 r.p.m. for 10 min. The supernatant was removed and the cells were precipitated with 10% trichloroacetic acid. Precipitates were harvested on GF/C filters under suction and washed with 5% trichloroacetic acid and industrial methylated spirit. The acid-insoluble radioactivity was determined by liquid scintillation counting. For each condition three incubations were carried out in duplicate and the data are shown as means ± S.E.M. of the counts per min incorporated per 10⁵ cells. Significances of differences between rates of protein synthesis were calculated by paired t test and the *P*-values are indicated in the figure legends.

Preparation of cell extracts and analysis by immunoblotting.

Cytoplasmic extracts were prepared for immunoblotting by washing 1 × 10⁷ cells in 1 ml of PBS and resuspending the pellet in 500 µl of cell lysis buffer (50 mM MOPS, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate) and 10 µl of 10% NP40 containing the following phosphatase and proteinase inhibitors: one tablet of protease inhibitors (Complete Mini, EDTA-free protease inhibitor cocktail, Roche) per 10 ml of cell lysis buffer, 2 mM sodium vanadate, 1 µM microcystin, 7 mM 2-mercaptoethanol and 1 mM phenylmethylsulphonylfluoride. After centrifugation for 5 min at 9200 × *g* to pellet the nuclei the cytoplasmic extracts were analysed by SDS gel electrophoresis, loading equal amounts of protein in each lane of the gel (10–20 µg of protein per sample). The proteins were transferred to poly(vinylidene difluoride) membranes and the blots were blocked and then incubated with the appropriate primary antibodies. Antibodies against eIF4G1 (central region) and eIF4E were kindly provided by Dr Simon Morley. Additional antibodies against eIF4G1 (N-terminal region), phospho-eIF4G1 (Ser¹¹⁰⁸), total Akt, phospho-Akt (Ser⁴⁷³), total Mek1/2, phospho-Mek1/2 (Ser^{217/221}), total Erk1/2, phospho-Erk1/2 (Thr²⁰²/Tyr²⁰⁴), total p38 MAPK, phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), total cdc2 and phospho-cdc2 (Thr¹⁶¹) were purchased from Cell Signaling Technology. Antibodies against 4E-BP1 and α-tubulin were purchased from Santa Cruz and Sigma, respectively. All blots were developed with horseradish peroxidase-linked secondary antibodies using enhanced chemiluminescence (Cell Signaling Technology). Blots were analysed for α-tubulin as a loading control.

m⁷GTP-Sepharose affinity chromatography. 50% (v/v) m⁷GTP-Sepharose 4B resin (Amersham) was washed in PBS and resuspended in cell lysis buffer. 50 µl of 50% (v/v) m⁷GTP-Sepharose 4B resin was added to cell extracts containing 170 µg of protein and incubated for 25 min at 4°C on an Eppendorf tube shaker. The beads and bound proteins were then isolated by centrifugation in a microcentrifuge at 4°C and washed three times with 200 µl cell lysis buffer (without detergent). The pellets were resuspended in 40 µl 2 × SDS sample buffer, boiled and centrifuged, and gels were loaded with 20 µl per lane. After electrophoresis 4E-BP1 and eIF4E were detected by immunoblotting as described above.

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Supplementary Information accompanies the paper on the Cell Death and Differentiation website (<http://www.nature.com/cdd>)