factor. Activation of Ras by G-protein-coupled receptors may be of particular relevance in tissues, such as cardiac muscle and the central nervous system[11,22], in which Ras activation may be associated with gene expression induced in the hypertrophic growth of muscle or by neurotransmission[23–25]. □

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1. Winitz, S. *et al. J. biol. Chem.* **268,** 19196–19199 (1993).
2. Mattingly, R. R., Sorisky, A., Brann, M. R. & Macara, I. G. *Molec. cell. Biol.* **14,** 7943–7952 (1994).
3. Egan, S. E. *et al. Nature* **363,** 45–51 (1993).
4. Buday, L. & Downward, J. *Cell* **73,** 611–620 (1993).
5. Martegani, E. *et al. EMBO J.* **11,** 2151–2157 (1992).
6. Shou, C., Farnsworth, C. L., Neel, B. G. & Feig, L. A. *Nature* **358,** 351–354 (1992).
7. Boguski, M. S. & McCormick, F. *Nature* **366,** 643–654 (1993).
8. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L. & Moolenaar, W. H. *Proc. natn. Acad. Sci. U.S.A.* **90,** 1257–1261 (1993).
9. van Biesen, T. *et al. Nature* **376,** 781–784 (1995).
10. Downward, J., Graves, J. D., Warne, P. H., Rayter, S. & Cantrell, D. A. *Nature* **346,** 719–723 (1990).
11. Farnsworth, C. L. *et al. Nature* **376,** 524–527 (1995).
12. Guerrero, C. *et al. Oncogene* **12,** 1097–1107 (1996).
13. Shou, C., Wurmser, A., Ling, K., Barbacid, M. & Feig, L. A. *Oncogene* **10,** 1887–1893 (1995).
14. Cen, H., Papageorge, A. G., Vass, W. C., Zhang, K. & Lowy, D. R. *Molec. cell. Biol.* **13,** 7718–7724 (1993).
15. Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M. & Lefkowitz, R. J. *J. biol. Chem.* **270,** 17148–17153 (1995).
16. Wolfman, A. & Macara, I. G. *Science* **248,** 67–69 (1990).
17. Gutkind, J. S., Novotny, E. A., Brann, M. R. & Robbins, K. C. *Proc. natn. Acad. Sci. U.S.A.* **88,** 4703–4707 (1991).
18. Koch, W. J., Hawes, B. E., Allen, L. F. & Lefkowitz, R. J. *Proc. natn. Acad. Sci. U.S.A.* **91,** 12706–12710 (1994).
19. Crespo, P., Xu, N., Simmonds, W. F. & Gutkind, J. S. *Nature* **369,** 418–420 (1994).
20. Faure, M., Voyno-Yasenetskaya, T. A. & Bourne, H. R. *J. biol. Chem.* **269,** 7851–7854 (1994).
21. Porfiri, E. & McCormick, F. *J. biol. Chem.* **271,** 5871–5877 (1996).
22. LaMorte, V. J. *et al. J. biol. Chem.* **269,** 13490–13496 (1994).
23. Hanley, M. R. *Nature* **340,** 97 (1989).
24. Ramirez, M. T., Post, G. R., Sulakhe, P. V. & Brown, J. H. *J. biol. Chem.* **270,** 8446–8451 (1995).
25. Thorburn, A. *et al. J. biol. Chem.* **268,** 2244–2249 (1993).
26. Posada, J. & Cooper, J. A. *Science* **255,** 212–215 (1992).
27. Mattingly, R. R., Wasilenko, W. J., Woodring, P. J. & Garrsion, J. C. *J. biol. Chem.* **267,** 7470–7477 (1992).
28. Linnemann, D. in *A Dissection and Tissue Culture Manual of the Nervous System* (ed. Shahar, A. *et al.*) 75–76 (Alan R. Liss, New York, 1989).
29. Graber, S. G., Figler, R. A., Kalman-Maltese, V. K., Robishaw, J. D. & Garrison, J. C. *J. biol. Chem.* **267,** 13123–13126 (1992).
30. Brondyk, W. H., McKiernan, C. J., Burstein, E. S. & Macara, I. G. *J. biol. Chem.* **268,** 9410–9415 (1993).

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Role for c-Abl tyrosine kinase in growth arrest response to DNA damage

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THE c-Abl protein tyrosine kinase is activated by certain DNA-damaging agents[1], and its overexpression causes arrest in the G1 phase of the cell cycle by a mechanism dependent on the tumour-suppressor protein p53 (refs 2–4). Here we investigate the possible role of c-Abl in growth arrest induced by DNA damage. Transient transfection experiments using wild-type or inactivated c-Abl show that both induce expression of p21, an effector of p53, but only wild-type c-Abl downregulates the activity of the cyclin-dependent kinase Cdk2 and causes growth arrest. Exposure to ionizing radiation of cells that stably express active or inactive c-Abl is associated with induction of c-Abl/p53 complexes and p21 expression. However, cells expressing the dominant-negative c-Abl mutant and cells lacking the c-*abl* gene are impaired in their ability to downregulate Cdk2 or undergo G1 arrest in response to ionizing radiation. We also show that expression of c-Abl kinase in *p21*$^{-/-}$, but not in *p53*$^{-/-}$, cells results in downregulation of Cdk2. Our results suggest that c-Abl kinase contributes to the regulation of growth arrest induced by ionizing radiation by a p53-dependent, p21-independent mechanism.

To determine whether c-Abl affects the function of p53, we co-transfected MCF-7 cells with a construct containing the luciferase gene driven by a p53 enhancer from the *MDM2* promoter[5], and vectors expressing wild-type c-Abl, a kinase-inactive K(290)R mutant[2] or a kinase-active mutant, designated ΔProl, which is deleted at the p53-binding domain[4]. Co-transfections of the reporter with wild-type or kinase-inactive c-Abl(K-R), but not c-AblΔProl, resulted in induction of luciferase activity (Fig. 1*a*). By contrast, c-Abl expression had no detectable effect on activation of an SV40–luciferase construct (Fig. 1*a*). As transcription of *p21* is regulated by p53 (ref. 6), we investigated whether transfection of c-Abl induces p21. We found that p21 expression increased in the c-Abl and c-Abl(K-R), but not the c-AblΔProl, transfectants (Fig. 1*b*). Despite induction of p21, which is an inhibitor of Cdk2

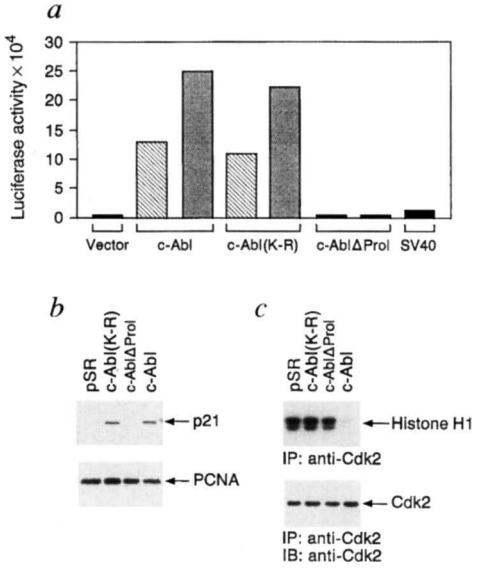

FIG. 1 Overexpression of kinase active c-Abl downregulates Cdk activity. *a*, MCF-7 cells were transfected with 2 μg p53-enhancer–luciferase plasmid (mdm2NA-Luc) and: (1) 8 μg control vector pSRaMSVtkNeo; (2) 5 and 8 μg c-*abl* vector; (3) 5 and 8 μg c-*abl*(K-R) vector; and (4) 5 and 8 μg c-*abl*ΔProl vector. Cells were also transfected with 2 μg SV40-promoter–luciferase plasmid (pGL2-control vector; Promega) and 8 μg c-*abl* vector. Luciferase activity was measured and normalized for protein concentration to that for control vector. *b*, MCF-7 cells were transfected with 8 μg control, c-*abl*, c-*abl*(K-R) or c-*abl*ΔProl vector. Cell lysates were immunoblotted with anti-p21 (WAF1, Ab-1: Oncogene Science) (top) and anti-PCNA (PC10; Santa Cruz Biotechnology) (bottom). *c*, Cell lysates from the transient transfectants were immunoprecipitated (IP) with anti-Cdk2 (M2, Santa Cruz Biotechnology), followed by histone H1 kinase assay (top) or immunoblotting (IB) with anti-Cdk2 (bottom).
METHODS. Transient transfections were done with Lipofectamine (GIBCO–BRL). Cells were collected 48 h after transfection. Luciferase was assayed with an enhanced luciferase assay kit (1800K, Analytical Luminescence). Histone H1 kinase was assayed as described[16].

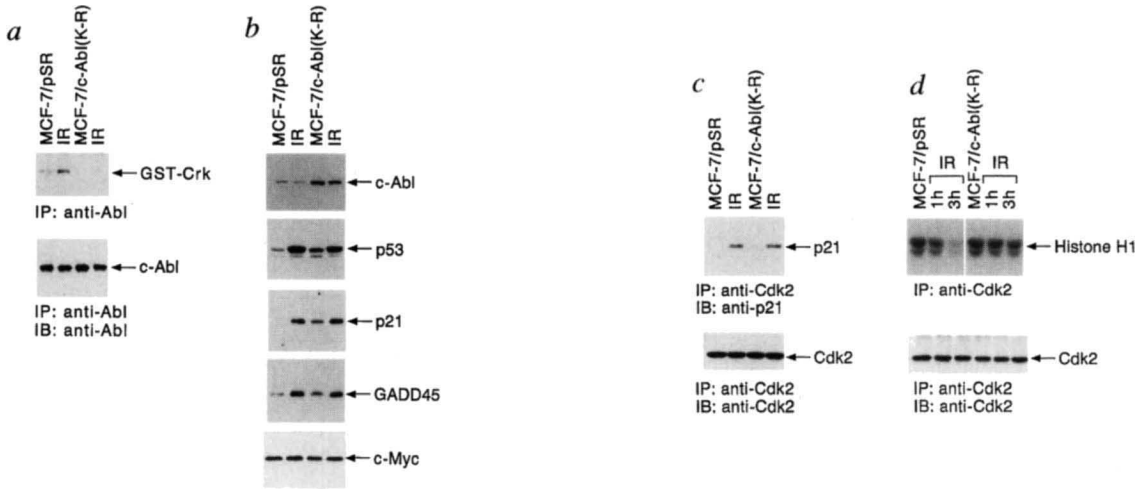

FIG. 2 c-Abl kinase activity regulates irradiation (IR)-induced inhibition of Cdk2. *a*, MCF-7 cells stably transfected with null pSR vector or c-*abl*(K-R) were treated with 5 Gy IR and collected after 3 h. Nuclei were isolated and nuclear proteins imunoprecipitated with anti-Abl (Ab-3; Oncogene Science) as described[1]. Immune-complex kinase was assayed using a glutathione-S-transferase (GST)–Crk (120–225) fusion protein as substrate (top). Anti-Abl immunoprecipitates were also analysed by immunoblotting with anti-Abl (bottom). *b*, Lysates were immunoblotted with anti-Abl, anti-p53 (Ab-6; Oncogene Science), anti-p21, anti-GADD45 (AT-26; Santa Cruz Biotechnology) and anti-c-Myc (9E10; Santa Cruz Biotechnology). *c*, Lysates were immunoprecipitated with anti-Cdk2. Precipitates were analysed by immunoblotting with anti-p21 and anti-Cdk2. *d*, Anti-Cdk2 immunoprecipitates were analysed by histone H1 kinase assay (top) or immunoblotting with anti-Cdk2 (bottom).
METHODS. Human MCF-7 breast cancer cells ($p53^{+/+}$)[17] were treated with 5 Gy IR at room temperature with a Gammacell 1000 (Atomic Energy of Canada) with a ^{137}Cs source emitting at a fixed dose rate of 0.76 Gy min^{-1}. Lysates were prepared in 0.5% NP-40 lysis buffer as described[1].

(refs 7–10), by both wild-type c-Abl and c-Abl(K-R), Cdk2 was downregulated only in cells transfected with wild-type c-Abl (Fig. 1*c*), which correlates with the ability of wild-type c-Abl, but not c-Abl(K-R) or c-AblΔProl, to inhibit growth in fibroblasts[2,3] and MCF-7 cells (data not shown). These findings suggest that the kinase activity and p53-binding domain of c-Abl are involved in downregulation of Cdk2 and thus in growth arrest, and that such effects are not exclusively mediated by activation of p21.

To determine the involvement of c-Abl in DNA-damage-dependent growth arrest, we prepared MCF-7 cells stably expressing the dominant-negative c-Abl(K-R)[2], which effectively inhibits the increase in c-Abl kinase activity induced by ionizing radiation on control cells (Fig. 2*a*). Irradiation of MCF-7/pSR or MCF-7/c-Abl(K-R) cells caused an increase in p53, p21 and GADD45, but not in c-Myc (which is not dependent on p53) (Fig. 2*b*); there was also increased binding of p21 to Cdk2 in MCF-7/pSR and in MCF-7/c-Abl(K-R) cells (Fig. 2*c*). Although Cdk2 was downregulated in irradiated MCF-7/pSR cells, there was little effect on Cdk2 activity in MCF-7/c-Abl(K-R) cells (Fig. 2*d*). These results indicate that c-Abl kinase is not required for transactivation of p21 and GADD45 by p53 in response to DNA damage, but is required for Cdk2 downregulation.

The role of c-Abl in growth arrest induced by ionizing radiation was tested by the ability of irradiated MCF-7/pSR and MCF-7/c-Abl(K-R) cells to reduce the number of cells in S phase. Using bromo-deoxyuridine (BrdU) labelling and bivariate fluorescence-activated cell-sorting (FACS) analysis, we found that more irradiated MCF-7/pSR cells arrested in G1 compared with controls (Fig. 3*a*). Only 15% of cells were in S phase 24 h after receiving 5 Gy radiation compared with untreated cells. However, irradiated MCF-7/c-Abl(K-R) cells were less affected, with the S-phase population being 45% of untreated samples (Fig. 3*a*).

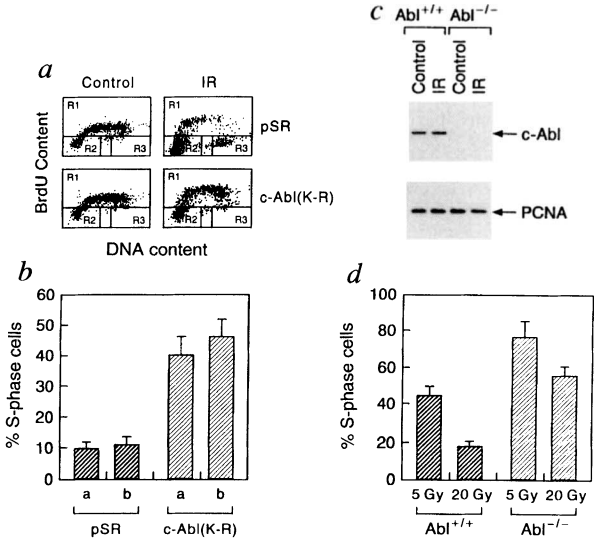

FIG. 3 c-Abl kinase activity regulates irradiation-induced growth arrest. *a*, Representative two-dimensional FACS analysis of MCF-7/pSR and MCF-7/c-Abl(K-R) cells after exposure to 0 or 5 Gy irradiation. Synchronized cells were irradiated and the percentage of cells in S phase was assessed at 24 h. Boxes labelled R1, R2 and R3 represent S, G1 and G2/M phase cells, respectively. *b*, Percentage of cells entering S phase after irradiation relative to control unirradiated cells. Results are expressed as the mean ± s.e.m. of 6 experiments for each of two (a, b) independently selected clones. *c*, C57BL/6 wild-type ($abl^{+/+}$) and $abl^{-/-}$ MEFs were exposed 0 or 5 Gy and collected at 3 h. Cell lysates were immunoblotted with anti-Abl and anti-PCNA antibodies. *d*, $abl^{+/+}$ and $abl^{-/-}$ MEFs were exposed to 0, 5 or 20 Gy irradiation. Results (mean ± s.e.m. of 6 experiments) are expressed as the percentage of cells entering S phase after irradiation relative to unirradiated cells.
METHODS. MCF-7/pSR and MCF-7/c-Abl(K-R) cells were blocked at G1/S phase by a 12-h treatment with 10 μM aphidicolin. Cells were then washed, irradiated and fed with fresh medium containing 10% FCS. Asynchronous populations of $abl^{+/+}$ and $abl^{-/-}$ MEFs[11] were exposed to IR and the percentage of cells in S phase assessed at 20 h. BrdU was added 30 min before collection. Cells were stained for DNA content with propidium iodide and for DNA synthesis with a fluorescein-conjugated anti-BrdU antibody (Boehringer Mannheim).

Similar results were obtained with two independently isolated MCF-7/c-Abl(K-R) clones (Fig. 3*b*). To confirm the link between c-Abl and radiation induced growth arrest, we studied mouse embryo fibroblasts (MEFs) deficient in c-Abl (*abl*$^{-/-}$ mice with targeted disruption of the c-*abl* gene)[11] (Fig. 3*c*). Wild-type MEFs after 5 Gy radiation had an S-phase population that was 45% of that for untreated cells (Fig. 3*d*). By contrast, irradiated *abl*$^{-/-}$ MEFs had more than 70% of cells in S phase compared with controls (Fig. 3*d*). Exposure of the wild-type and *abl*$^{-/-}$ MEFs to 20 Gy radiation also demonstrated partial inhibition of arrest in G1 in the *abl*$^{-/-}$ cells. These findings together indicate that the c-Abl kinase function is necessary for radiation-induced G1 arrest.

As c-Abl and p53 bind together *in vitro*[4], we tested whether they were associated in irradiated cells. No p53 was detected in anti-Abl immunoprecipitates from MCF-7/pSR cells that had not been irradiated (Fig. 4*a*), but binding of c-Abl and p53 was evident at 30 min and maximal at 3–5 h after irradiation (Fig. 4*a*, and data not shown). The small amount of constitutive binding of c-Abl to p53 found in MCF-7/c-Abl(K-R) cells was stimulated by irradiation (Fig. 4*a*).

To investigate further the role of p53 in growth regulation by c-Abl, we transfected *p53*$^{-/-}$ fibroblasts with wild-type c-Abl and c-Abl(K-R). Analysis of p21 levels and Cdk2 activity failed to reveal any effect with either vector (Fig. 4*b*, and data not shown). However, Cdk2 was downregulated in *p53*$^{+/+}$ fibroblasts after transfection with wild-type c-Abl, but not c-Abl(K-R) (Fig. 4*b*). These results show that c-Abl kinase inhibits Cdk2 by a p53-dependent mechanism. We also investigated the effect on Cdk2 activity of irradiating *abl*$^{-/-}$ cells and found that this did not decrease as much as in wild-type MEFs (Fig. 4*c*). In *p21*$^{-/-}$ fibroblasts, it was found that p21 only partially mediates the action of p53 in arresting radiation-damaged cells in G1 (refs 12, 13). In support of our previous findings, expression of c-Abl(K-R) had no effect on Cdk2 activity in *p21*$^{-/-}$ or *p21*$^{+/+}$ cells (Fig. 4*d*); whereas transfection of wild-type c-Abl into *p21*$^{-/-}$ cells inhibited Cdk2 (Fig. 4*d*) and growth (data not shown), indicating that these effects of c-Abl depend on p53 but not p21. It is possible, however, that growth arrest following transfection with c-Abl inhibits Cdk2 activity.

Our results obtained using a dominant-negative c-Abl mutant suggest that c-Abl regulates growth in response to genotoxic stress. Because c-Abl associates with the retinoblastoma protein Rb[14] and overexpression of kinase-defective c-Abl abrogates Rb-induced growth arrest[15], mutant c-Abl may prevent G1 arrest in response to DNA damage by inactivating Rb. But as the partial loss of G1 arrest in irradiated cells expressing c-Abl(K-R) is associated with a block in Cdk2 downregulation, the effect probably occurs upstream of Rb. We have demonstrated that c-Abl functions in the cellular response to DNA damage through p53-dependent pathways, confirmed by the failure of the c-AblΔProl mutant to bind p53[4] and downregulate Cdk2 and arrest growth. We have also shown that the kinase-defective c-Abl(K-R) mutant induces p53 transactivation but not G1 arrest, indicating that stimulation of p53 transactivation function by c-Abl is not sufficient for this response. Although c-Abl stimulates the transcriptional activity of p53, as measured by expression of its target gene *p21*, a different mechanism is used by p21 in growth arrest[10] because c-Abl-mediated transactivation is kinase-independent and Cdk2 downregulation and growth arrest by the c-Abl kinase are p21-independent. These results support a novel c-Abl/p53 dependent, but p21-independent, mechanism for the regulation of Cdk2. □

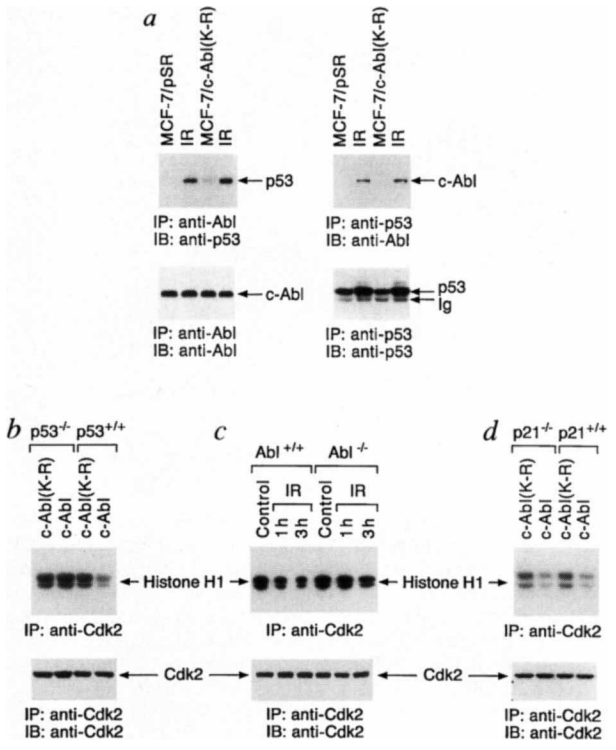

FIG. 4 c-Abl kinase downregulates Cdk2 activity by a p53-dependent, p21-independent mechanism. *a*, Lysates were immunoprecipitated with anti-Abl (left) or anti-p53 (right) and the precipitates analysed by immunoblotting with the indicated antibodies. Ig, immunoglobulin. *b*, MEFs (*p53*$^{-/-}$, *p53*$^{+/+}$)[18] were transfected with the c-*abl* or c-*abl*(K-R) vectors and collected at 48 h. c, MEFs (*abl*$^{+/+}$, *abl*$^{-/-}$)[11] were exposed to 0 or 5 Gy and collected at the indicated times. *d*, MEFs (*p21*$^{-/-}$, *p21*$^{+/+}$)[12] were transfected with the c-*abl* or c-*abl*(K-R) vectors and collected at 48 h. Cell lysates were immunoprecipitated with anti-Cdk2 and the precipitates assayed for histone H1 kinase activity (top) or immunoblotted with anti-Cdk2 (bottom) as described for Fig. 1.

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1. Kharbanda, S. *et al. Nature* **376,** 785–788 (1995).
2. Sawyers, C. L., McLaughlin, J., Goga, A., Havilik, M. & Witte, O. *Cell* **77,** 121–131 (1994).
3. Mattioni, T., Jackson, P. K., Bchini-Hooft van Huijsduijnen, O. & Picard, D. *Oncogene* **10,** 1325–1333 (1995).
4. Goga, A. *et al. Oncogene* **11,** 791–799 (1995).
5. Barak, Y., Gottlieb, E., Juven-Gershon, T. & Oren, M. *Genes Dev.* **8,** 1739–1749 (1994).
6. El-Deiry, W. *et al. Cell* **75,** 817–825 (1993).
7. Gu, Y., Turck, C. & Morgan, D. *Nature* **366,** 707–710 (1993).
8. Harper, J. W. *et al. Molec. Biol. Cell* **6,** 387–400 (1995).
9. Xiong, Y. *et al. Nature* **366,** 701–704 (1993).
10. Dulic, V. *et al. Cell* **76,** 1013–1023 (1994).
11. Tybulewicz, V. L. J., Crawford, C. E., Jackson, P. K., Bronson, R. T. & Mulligan, R. C. *Cell* **65,** 1153–1163 (1991).
12. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. *Cell* **82,** 675–684 (1995).
13. Brugarolas, J. *et al. Nature* **377,** 552–557 (1995).
14. Welch, P. J. & Wang, J. Y. J. *Cell* **75,** 779–790 (1993).
15. Welch, P. J. & Wang, J. Y. J. *Molec. cell. Biol.* **15,** 5542–5551 (1995).
16. Yuan, Z.-M., Kharbanda, S. & Kufe, D. *Biochemistry* **34,** 1058–1063 (1995).
17. Bartek, J., Vojtesek, B., Grand, R. J. A., Gallimore, P. H. & Lane, D. P. *Oncogene* **7,** 101–108 (1992).
18. Lowe, S. W., Jacks, T., Housman, D. E. & Ruley, H. E. *Proc. natn. Acad. Sci. U.S.A.* **91,** 2026–2030 (1994).

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