regulating p53 stability during stress responses, it may also be involved in controlling basal p53 levels to an extent that depends on cell type and context. Defective Mdm2 may allow p53 to accumulate uncontrollably, explaining why p53 in the absence of Mdm2 becomes lethal to the developing embryo[28,29]. □

Methods

Cells and transfection. 6×10^5 HeLa or H1299 cells were transfected by the calcium phosphate method using 2 μg p53 expression plasmid and 5 μg *mdm2* plasmid. T47D cells were transfected with lipofectamine (Gibco BRL) using 3 μg of *mdm2* plasmid DNA. For western blotting and immunofluorescent staining, cells were collected 26 h after transfection. Expression plasmids for p53 were: pCMV-Neo-Bam-p53 (human WT p53) pRCp53Gln14Ser19 (mutant human p53)[10] and p53Δ13–52 (mutant murine p53 lacking residues 13 to 52). Gal4-p53 fusion constructs were pGal4p53 and pGal4p53(1–42) (ref. 12) Mdm2 expression plasmids *X2* and *ΔXM* have been described[6,9]

Pulse-chase analysis. H1299 cells were metabolically labelled for 10 min 26 h post-transfection as described[6]. Labelled cells were washed twice in PBS and then maintained in non-radioactive medium for 0, 60 or 180 min before collecting. Immunoprecipitation has been described[6]. p53 was immunoprecipitated with PAb421 monoclonal antibody. Precipitated proteins were resolved by SDS–PAGE and the gel fluorographed and exposed to X-ray film.

Northern blotting and RT-PCR. mRNA was prepared from transfected H1299 cells. 10 μg mRNA was northern blotted using a p53 probe (nucleotides −25 to 656 of human p53 cDNA, radiolabelled with ^{32}P by random priming). For polymerase chain reaction with reverse transcription (RT-PCR), total mRNA was prepared from each sample of ML-1 cells and used as a template for reverse transcription. Amounts of cDNA were normalized by 22 cycles of PCR using specific β-actin primers (sense, 5′-GAGCACCCTGTGCTGCTCACCGAG-3′; antisense, 5′-GTGGTGGTGAAGCTGTAGCCACGCT-3′). Using the same amount of total cDNA, p53 cDNA was quantified by 26 cycles of PCR with primers specific for human p53 (sense, 5′-AGTGGATCCAGACTGCCTTCC-GGGTCACTG-3′; antisense, 5′-GCGGATCCTAGGGCACCACCACACTAT-3′). Conditions for PCR were: 94 °C for 30 s, 60 °C for 30 s, then 72 °C for 60 s.

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1. Gottlieb, T. & Oren, M. p53 in growth control and neoplasia. *Biochim. Biophys. Acta* **1287,** 77–102 (1996).
2. Shen, Y. & Shenk, T. E. Viruses and apoptosis. *Curr. Opin. Gene. Dev.* **5,** 105–111 (1995).
3. Picksley, S. M. & Lane, D. P. The p53–mdm2 autoregulatory feedback loop—a paradigm for the regulation of growth control by p53? *Bioessays* **15,** 689–690 (1993).
4. Chen, J. D., Lin, J. Y. & Levine, A. J. Regulation of transcription functions of the p53 tumor suppressor by the *mdm-2* oncogene. *Mol. Med.* **1,** 141–142 (1995).
5. Chen, C. Y. *et al.* Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc. Natl Acad. Sc. USA* **91,** 2684–2688 (1994).
6. Haupt, Y., Barak, Y. & Oren, M. Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.* **15,** 1596–1606 (1996).
7. Chen, J. D.,Wu, X., Lin, J. Y. & Levine, A. J. mdm-2 inhibitis the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol. Cell. Biol.* **16,** 2445–2452 (1996).
8. Perry, M. E., Piette, J., Zawadzki, J. A., Harvey, D. & Levine, A. J. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *Proc. Natl Acad. Sci. USA* **90,** 11623–11627 (1993).
9. Barak, Y., Gottlieb, E., Juven-Gershon, T. & Oren, M. Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev.* **8,** 1739–1749 (1994).
10. Lin, J. Y., Chen, J. D., Elenbaas, B. & Levine, A. J. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.* **8,** 1235–1246 (1994).
11. Hinds, P. W. *et al.* Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells—a comparison of the hot spot mutant phenotype. *Cell Growth Diff.* **1,** 571–580 (1990).
12. Unger, T., Nau, M. M., Segal, S. & Minna, J. D. p53—a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.* **11,** 1383–1390 (1992).
13. Oren, M., Reich, N. C. & Levine, A. J. Regulation of the cellular p53 tumor antigen in teratocarcinoma cells and their differentiated progeny. *Mol. Cell. Biol.* **2,** 443–449 (1982).
14. Yonish-Rouach, E. *et al.* Induction of apoptosis by transiently transfected metabolically stable wt p53 is transformed cell lines. *Cell Death Differ.* **1,** 39–47 (1994).
15. Palombella, V. J., Rando, O. J., Goldberg, A. L. & Maniatis, T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78,** 773–785 (1994).
16. Maki, C. J., Huibregtse, J. M. & Howley, P. M. *In vivo* ubiquitination and proteasome-mediated degradation of p53. *Cancer Res.* **56,** 2649–2654 (1996).
17. Kubbutat, M. H. & Vousden, K. H. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* **17,** 460–468 (1997).
18. Gottlieb, E., Lindner, S. & Oren, M. Relationship of sequence-specific transactivation and p53-regulated apoptosis in interleukin 3-dependent hematopoietic cells. *Cell Growth Diff.* **7,** 301–310 (1996).
19. Lane, D. P. The regulation of p53 function: Steiner award lecture. *Int. J. Cancer* **57,** 623–627 (1994).
20. Bartek, J., Iggo, R., Gannon, J. & Lane, D. P. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* **5,** 893–899 (1990).
21. Martin, K. *et al.* Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* **375,** 691–694 (1995).
22. Xiao, Z. X. *et al.* Interaction between the retinoblastoma protein and the oncoprotein Mdm2. *Nature* **375,** 694–698 (1995).
23. Momand, J. & Zambetti, G. P. Analysis of the proportion of p53 bound to mdm-2 in cells with defined growth characteristics. *Oncogene* **12,** 2279–2289 (1996).
24. Kastan, M. B., Onyekwere, O., Sindransky, D., Vogelstein, B. & Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51,** 6304–6311 (1991).
25. Fiscella, M. *et al.* Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene* **8,** 1519–1528 (1993).
26. Scheffner, M., Huibregtse, J. M., Vierstra, R. D. & Howley, P. M. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75,** 495–505 (1993).
27. Otto, A. & Deppert, W. Upregulation of mdm-2 expression in Meth A tumor cells tolerating wild-type p53. *Oncogene* **8,** 2591–2603 (1993).
28. Jones, S. N., Roe, A. E., Donehower, L. A. & Bradley, A. Rescue of embryonic lethality in mdm2-deficient mice by absence of p53. *Nature* **378,** 206–208 (1995).
29. Montes de Oca Luna, R., Wagner, D. S. & Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378,** 203–206 (1995).

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Regulation of p53 stability by Mdm2

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The tumour-suppressor p53 is a short-lived protein that is maintained at low, often undetectable, levels in normal cells. Stabilization of the protein in response to an activating signal, such as DNA damage, results in a rapid rise in p53 levels and subsequent inhibition of cell growth[1]. Tight regulation of p53 function is critical for normal cell growth and development, and one mechanism by which p53 function is controlled is through interaction with the Mdm2 protein[2–4]. Mdm2 inhibits p53 cell-cycle arrest and apoptic functions[5,6] and we show here that interaction with Mdm2 can also result in a large reduction in p53 protein levels through enhanced proteasome-dependent degradation. Endogenous levels of Mdm2 are sufficient to regulate p53 stability, and overexpression of Mdm2 can reduce the amount of endogenous p53. Because *mdm2* is transcriptionally activated by p53 (refs 7, 8), this degradative pathway may contribute to the maintenance of low p53 concentrations in normal cells. Furthermore, mechanisms regulating the Mdm2-induced degradation of p53 may play a role in controlling the extent and duration of the p53 response.

Mdm2 plays a critical role in regulating p53 function, as shown by the observation that deletion of the *mdm2* gene is lethal in mice during early embryogenesis only in animals carrying a functional *p53* gene[9,10]. Simultaneous deletion of both *mdm2* and *p53* gave rise to mice that developed normally, illustrating that Mdm2 is essential for negative regulation of the growth-inhibitory activities of p53 during development. Although Mdm2 is clearly not necessary for development in the absence of p53, only the amino-terminal domain of Mdm2 is necessary to inhibit p53 transcriptional and G1-arrest functions[6], suggesting that the full-length protein may carry out additional functions. These may be related to other properties of Mdm2, such as interactions with the tumour-suppressor protein RB (ref. 11) and the transcription factor E2F (ref. 12).

We have previously described a *p53* mutant, *p53ΔI*, which

produces a protein lacking the amino acids constituting conserved region I (residues 13–19)[13]. Although this protein failed to interact with Mdm2 in an *in vitro* assay, it retained wild-type transcriptional activity[13] which, in contrast to wild-type p53, is not inhibited by coexpression of Mdm2 (unpublished observations). Transient transfection assays using wild-type p53 and the p53ΔI mutant in Saos-2, a p53-null human tumour cell line, showed that p53ΔI retained the ability to induce both cell-cycle arrest and apoptosis (data not shown), indicating that the interaction with Mdm2 is not essential for these functions of p53. Previous reports have ascribed the inhibition of p53 function by Mdm2 to the physical interaction between these two proteins, which masks the transactivation domain of p53. However, during the course of these studies we noted that co-transfections of Mdm2 with wild-type p53 reproducibly reduced the number of p53-positive cells detected by flow cytometry, although this was not evident with p53ΔI (Fig. 1a). This suggested that Mdm2 may be regulating p53 expression. To examine this more closely, steady-state p53 protein levels in the transfected cells were analysed by western blotting. Wild-type p53 was expressed to lower levels than the p53ΔI mutant protein, and after cotransfection of *mdm2* the levels of wild-type p53 were markedly reduced, although this was not apparent using the p53ΔI mutant (Fig. 1b). Expression of the transfected *mdm2* in these experiments was confirmed by western blotting (data not shown). Titration of the amount of cotransfected *mdm2* plasmid showed that increasing amounts of Mdm2 correlated with decreased levels of p53 (Fig. 1c). Similar analysis of several other p53 proteins carrying mutations within the N terminus of p53 extended the correlation between the ability of p53 to bind to Mdm2 and the abrogation of expression following cotransfection of *p53* and *mdm2* (Fig. 1d). The double point mutant p53 22/23, which fails to interact with Mdm2[14], behaved like p53ΔI in showing constant expression levels with or without Mdm2. p53Ala15 and p53Asp15, which have substitutions of the serine at residue 15 that is phosphorylated following activation of p53, potentially by DNA-activated protein kinase[15], behaved like wild-type p53 in showing strongly reduced protein levels following cotransfection with *mdm2*. Both of these mutants also retained some ability to interact with Mdm2 *in vitro* (N. J. Marson and K.H.V., unpublished data).

To assess whether the ability of Mdm2 to modulate the amount of p53 protein expressed in cells is mediated at the transcriptional level, northern blots of RNA extracted from transiently transfected cells were carried out (Fig. 2a). This analysis showed clearly that under conditions where the p53 protein levels are dramatically reduced following cotransfection with *mdm2*, there is no discernible reduction in the p53 messenger RNA levels. Similarly, the high expression of p53ΔI protein seen in the presence of exogenous Mdm2 is not due to increased transcription of this mutant. These results indicate that regulation of p53 levels by Mdm2 is through a post-transcriptional mechanism. The levels of p53 in normal cells are regulated by the rapid turnover of the protein, and activation of the p53 pathway following DNA damage results in increased amounts of p53, in part through a lengthening of the half-life of the protein. At least one protein, human papillomavirus E6, has been shown to abrogate efficiently the normal cellular p53 response by forming an interaction with p53 and targeting it for rapid ubiquitin-dependent degradation[16]. To determine whether Mdm2 has a similar effect on p53 stability, we analysed the half-life of p53 protein expressed in cells with or without Mdm2 (Fig. 2b). The half-life of transfected wild-type p53 in these human cells was approximately 7.3 h, consistent with previous estimates of the half-life of endogenous p53 in human epithelial cells[17], and transfection with *mdm2* resulted in the decreased stability of the wild-type p53 protein, reducing the half-life to 2.5 h. By contrast, the p53ΔI mutant protein remained stable during the 12-h chase and was not affected by coexpression of Mdm2. These results show that the interaction of Mdm2 with p53 results in an enhanced degradation of the p53 protein, thus reducing the steady-state levels of p53 detected

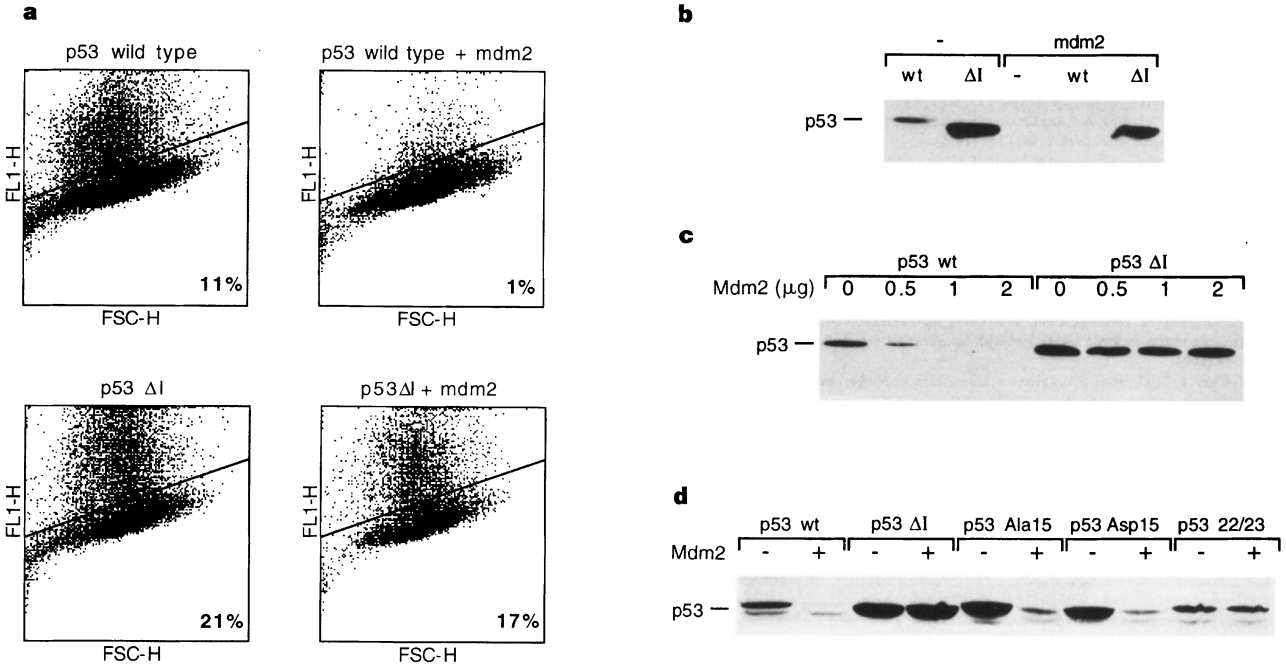

Figure 1 Mdm2 reduces expression of p53 protein. Steady-state levels of p53 protein in Saos-2 cells transiently transfected with wild-type *p53* or each *p53* mutant, with or without mouse *mdm2*. **a**, FACS analysis showing expression of p53 in cells following transient transfection with the indicated plasmids. Cells were labelled 24-h post-transfection with PAb421 and analysed by flow cytometry. p53-positive cells show increased fluorescence (above the line) compared with the untransfected population (below the line). The percentage of cells expressing p53 in each population is shown. **b**, Expression of wild-type p53 and p53ΔI in the presence or absence of exogenous Mdm2. **c**, Effects of increasing amounts of Mdm2 on the levels of wild-type p53 and p53ΔI protein. Wild-type (WT) *p53* and *p53*ΔI (1 μg), respectively, were cotransfected with the indicated amounts of *mdm2*. **d**, Levels of mutant p53 protein expression in the presence or absence of exogenous Mdm2. p53 protein was detected in lysate of transfected cells by western blot analysis using monoclonal antibody PAb1801.

by western blotting (Fig. 1).

The ability of Mdm2 to target p53 for degradation would have important implications for the mechanisms by which this regulator of p53 activity functions. The experiments described so far analysed the effects of exogenously transfected *mdm2*, and in order to determine whether physiological levels of endogenous Mdm2 were also able to carry out a similar function, we used mouse embryo fibroblasts derived from *p53*-deficient mice and from *mdm2/p53*-deficient mice[10,18]. Analysis of wild-type p53 expression in two independently derived lines of each genotype clearly showed that the presence of endogenous Mdm2 correlates with lower levels of p53 compared to cells lacking Mdm2 (Fig. 3). The p53ΔI mutant was expressed at equally high levels, regardless of the Mdm2 status of the cell, supporting the physiological relevance of the p53/Mdm2 interaction in the regulation of p53 stability. These results are also supported by the observation that p53ΔI has a longer half-life than the wild-type p53 protein in human cells in the absence of exogenous Mdm2 (Fig. 2), suggesting a role for endogenous Mdm2.

Having established that endogenously expressed Mdm2 can influence p53 protein levels, we sought to demonstrate an effect of Mdm2 on endogenous p53 levels and examined both cells expressing wild-type and cells expressing mutant p53 proteins. U2OS cells, which express wild-type p53 and mount an apparently normal p53 response following DNA damage, including activation of $p21^{WAF1/CIP1}$ and G1 arrest (data not shown), were transiently transfected with *mdm2*, DNA-damaged and collected for western-blot analysis at several time points (Fig. 4a). Endogenous p53 levels were low in these cells before damage and following DNA damage the control transfected cells showed a rapid accumulation of p53 protein over the 24-h time course. By contrast, *mdm2* overexpressing cells contained lower levels of p53 than the control cells before damage; this reduction of the already low levels of p53 protein was seen reproducibly in several experiments. Furthermore, *mdm2* expression significantly inhibited the accumulation of p53 following DNA damage (Fig. 4a). Interestingly, analysis of Mdm2 in these cells revealed a sharp decline in the expression of the exogenous protein after 8 h, consistent with our inability to generate a stable *mdm2*-overexpressing line. Provocatively, the reduction in *mdm2* expression in these cells exactly parallels the increase in p53 protein levels. These results therefore support a role for Mdm2 in both regulating basal levels of p53 expression and modulating the DNA-damage response. We also examined the effect of Mdm2 on p53 levels in C33A cells, which express high levels of a mutant p53 protein[19] that is still sensitive to degradation by Mdm2 following cotransfection in Saos-2 cells (data not shown). The p53 mutant expressed in these cells is unable to activate transcription of *mdm2*, and we considered the possibility that this inability to induce Mdm2 expression contributes to the accumulation of high levels of p53 mutant protein in these cells. We found that overexpression of wild-type Mdm2 in these cells resulted in a substantial decrease in the level of p53 protein expression in the transfected cells (Fig. 4b), an effect that was not seen following transfection of a control plasmid. A similar decrease in p53 protein expression was detected by flow cytometric analysis of C33A cells transfected with wild-type *mdm2* compared with cells transfected with an *mdm2* mutant, which encoded a protein that failed to target p53 for degradation (data not shown).

We next sought to identify the mechanism by which proteolytic degradation of p53 takes place. Several previous studies have suggested that p53 is normally ubiquitinated and degraded through the proteasome[20], although other proteolytic pathways may also be involved[21]. We therefore examined the effect of a specific inhibitor of the proteasome, lactacystin[22], on degradation of p53 by Mdm2 (Fig. 5). Inhibition of proteasome function in *mdm2*-expressing mouse embryo fibroblasts resulted in a clear defect in the ability to degrade exogenous p53 (Fig. 4a), although this effect was not seen in cells lacking Mdm2. Treatment of human cells expressing endogenous wild-type p53 and Mdm2 with this proteasome inhibitor resulted in enhanced stability of the endogenous p53 (Fig. 5b) and an inhibition of Mdm2-targeted p53 degradation could also be seen following cotransfection of these cells with *mdm2* and *p53* (Fig. 5b). These results strongly indicate that Mdm2 targets p53 for degradation through the proteasome, and suggests that Mdm2 is involved in the normal regulation of p53 stability.

Analysis of p53 mutants indicated that the regulation of p53 stability by Mdm2 is dependent on the interaction between the two proteins. Mdm2 is a large protein (491 amino acids for the human protein), of which only the N-terminal 100 amino acids are involved in the interaction with p53 (refs 4, 23). To determine whether additional Mdm2 sequences are required for the degradation of p53, we examined the effect of a previously described Mdm2 deletion mutant that retains the ability to interact with p53 and inhibit p53-induced cell-cycle arrest[6]. Although expression of wild-type Mdm2 resulted in the reduction of p53 protein levels (Fig. 6), expression of the Mdm2 mutant (Δ222–437) not only failed to target degradation of p53, but resulted in a significant elevation of p53 levels. These results show that binding of Mdm2 to p53 is not sufficient for

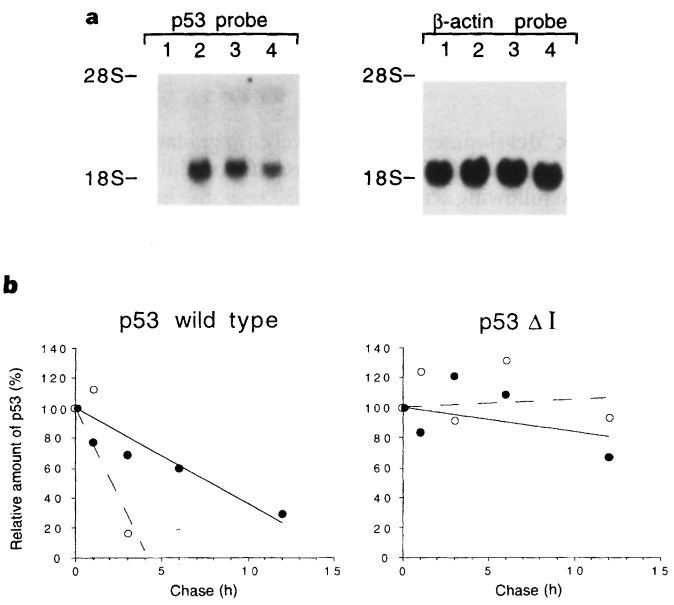

Figure 2 Mdm2 does not affect *p53* transcription, but reduces p53 protein stability. **a**, Northern blot analysis of total RNA extracted from Saos-2 cells transiently transfected with vector only (1), wild-type *p53* (2), wild-type *p53* plus mouse *mdm2* (3) or *p53ΔI* plus mouse *mdm2* (4). The filter was probed sequentially for expression of p53 and ß-actin. **b**, Pulse chase of p53 proteins in transiently transfected Saos-2 cells in the presence (empty circles, dashed line) or absence (filled circles, solid line) of exogenous mouse Mdm2. Amounts of labelled p53 protein detected at each time point were calculated relative to the amount present following 0-h chase and regression was calculated using Microsoft Excel.

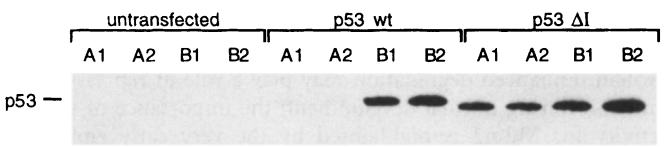

Figure 3 Expression of exogenous wild-type p53 and p53ΔI protein in two independent clones of *p53*-null (A1, A2) and *p53/mdm2* double-null (B1, B2) mouse embryo fibroblasts. Equal transfection efficiency of each line was confirmed by cotransfection of a ß-galactosidase expression vector (data not shown). p53 protein was detected after immunoprecipitation with PAb421 by western blot analysis using rabbit serum CM-1.

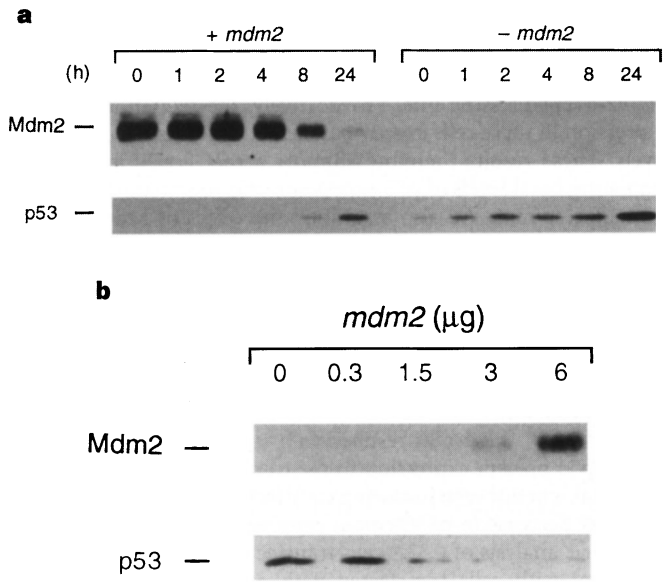

Figure 4 Mdm2 reduces endogenous p53 levels and delays DNA damage-induced p53 accumulation. **a**, U2OS cells transfected with 3 μg human *mdm2*-expressing plasmid or empty vector were treated with 5 nM actinomycin-D to induce a p53 response. At the indicated times successfully transfected cells were isolated using the Capture-Tec kit (Clontech) and p53 and Mdm2 protein expression was detected by western blotting using monoclonal antibodies DO-1 and IF2. **b**, C33A cells positively transfected with the indicated amounts of human *mdm2*-expressing plasmid or empty vector were analysed for p53 and Mdm2 protein expression by western blotting.

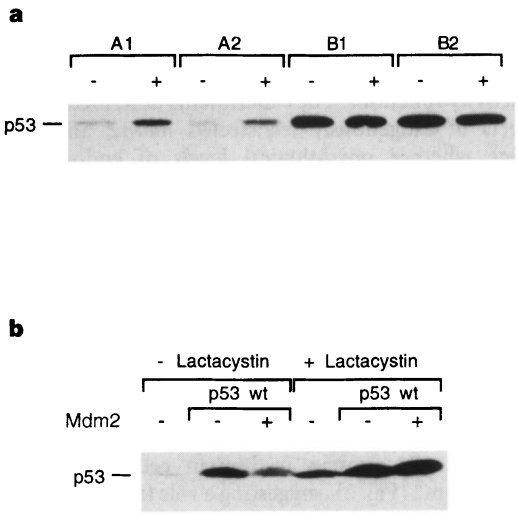

Figure 5 Inhibition of Mdm2-mediated degradation of p53 by lactacystin. **a**, Two independent clones of *p53*-null (A1, A2) and *p53/mdm2* double-null (B1, B2) mouse embryo fibroblasts were transfected with 1.5 μg wild-type *p53* plasmid. Cells were treated 24 h after transfection with medium containing 25 μM lactacystin (+) or left untreated. p53 protein was detected as described in Fig. 3. **b**, U2OS cells were cotransfected with wild-type p53 plasmid and mouse *mdm2*-expressing plasmid (+) or empty vector (–). Treatment with lactacystin is described in **a**. p53 protein was detected in cell lysates by immunoblot analysis using antibody PAb1801.

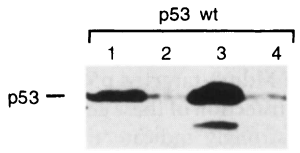

Figure 6 Binding of Mdm2 to p53 is not sufficient to induce degradation. U2OS cells were cotransfected with wild-type *p53* together with empty vector (1), human wild-type *mdm2* (2), human *mdm2Δ222-437* (3) encoding a mutant form of Mdm2 that can bind to p53, and mouse wild-type Mdm2 (4), respectively. p53 protein was detected in cell lysates by immunoblotting using PAb1801.

degradation and suggest a role for other domains of the Mdm2 protein. The Mdm2Δ222–437 mutant protein was very stably expressed, resulting in at least 20-fold more protein than the wild-type Mdm2 protein, as detected by western blotting in these cells (data not shown). Because these human cells express endogenous Mdm2, these results may indicate that at sufficiently high levels of overexpression, this mutant can act in a dominant negative manner, protecting the p53 from degradation by endogenous Mdm2 protein. Interestingly, expression of this mutant in cells expressing wild-type endogenous p53 resulted in an elevation of the normally very low p53 levels (data not shown), consistent with suggestion that the endogenous Mdm2 in these cells is contributing to p53 degradation, and that interference with wild-type Mdm2 function results in stabilization of the endogenous p53 protein.

The results reported here and in an accompanying paper[24] describe a new mechanism by which both mouse and human Mdm2 can regulate p53 function through degradation of the p53 protein. Enhanced degradation may play a role in repressing p53 function during normal development; the importance of such an activity for Mdm2 is highlighted by the very early embryonic lethality of Mdm2-deficient mice that retain wild-type p53. It is of interest to note that although binding of Mdm2 to p53 is apparently sufficient to inhibit transcriptional and cell-cycle arrest activities of p53, there is some evidence that this interaction may not inhibit all the apoptotic activities of the p53 protein[5]. Clearly, degradation of the p53 protein would prevent all p53-induced cell death and such a stringent inhibition of p53 function may be essential during embryonic development. Alternatively, degradation of p53 by Mdm2 may be an efficient mechanism for rapidly reversing cell-cycle arrest following activation of a p53 response in some cell types. Although most cells with wild-type p53 show a very rapid accumulation of the protein in response to activation, in some cells the high levels of p53 are maintained[25], whereas in other cell types p53 levels show a transient peak and then decline, corresponding with an ability to re-enter the cell cycle[26]. During the latter response, the rise in Mdm2 levels following p53 activation coincides with the drop in p53 levels[24,26,27], supporting a role for Mdm2-induced degradation of p53 during recovery from DNA damage. Normal human fibroblasts, however, show a sustained, apparently irreversible p53 response in which the increased amounts of p53 detected after DNA damage do not decline over time[25]. Interestingly, all the characteristics of a normal p53 response are maintained in these cells, including the enhanced expression of p53-inducible genes such as $p21^{WAF1/CIP1}$ and *mdm2* (S. Bates and K.H.V., unpublished data). It therefore seems likely that modification of either Mdm2 or p53, such as by phosphorylation, may protect from Mdm2-induced degradation under these conditions, thereby allowing a sustained p53 response.

The observation that expression of Mdm2, which is at least in part dependent on p53, regulates the stability of the p53 protein may provide some explanation for the enhanced stability of many p53 mutants often found in tumour cells, because these mutant proteins are transcriptionally inactive and therefore incapable of activating expression of the Mdm2 protein that would target

them for degradation. Finally, our study suggests a role for Mdm2 sequences distinct from those required for p53 binding, in targeting proteins for degradation, suggesting that other Mdm2-associated proteins, such as RB and E2F, may also be destabilized as a result of the interaction with Mdm2, or may modify the stability of p53 through their interaction with Mdm2. □

Methods

Plasmids, antibodies and proteasome inhibitor. Plasmids encoding mouse wild-type Mdm2 (pCOC Mdm2 X2)[5], human wild-type Mdm2 (pCHDM), human mutant Mdm2 Δ222–437 (pCHDM Δ222–437)[6], human wild-type p53 (pCB6 + p53Pro) and mutant p53 (pCB6 + p53ΔI (ref. 13), pcB6 + Ala15, pCB6 + Asp15 (ref. 28) and pCMV p53 22/23 (ref. 14)) under the control of the CMV promoter have been described previously. p53-specific monoclonal antibodies PAb1801, D0-1 and PAb421 and the Mdm2-specific antibody IF2 were purchased from Oncogene Science. The polyclonal rabbit serum CM-1 was from Novocastra. The proteasome inhibitor lactacystin was purchased from E. J. Corey.

Cells and transfections. Saos-2 (p53 null), U2OS (p53 wild type), C33A (p53 mutant) cells, and *p53*-null and *p53/mdm2* double-null mouse embryo fibroblasts (MEF) were maintained in DMEM supplemented with 10 or 15% fetal calf serum, respectively, and transiently transfected using the calcium phosphate precipitation method. Unless otherwise indicated, 1 μg wild-type *p53* or mutant *p53* plasmid were cotransfected with 2 μg Mdm2-encoding plasmid per 60-mm dish and cells were collected 24 h after transfection. Where appropriate, transfection efficiency was verified by cotransfection of 1.5 μg of CMV-driven β-galactosidase expression plasmid and measurement of enzymatic β-galactosidase activity. For studies of endogenous p53 levels, cells were cotransfected with 1.5 μg pHook and 1.5 μg β galactosidase expression plasmid and transfected cells were separated from untransfected cells using the Capture-Tec kit (Clontech).

Protein analysis. Western blotting, immunoprecipitation[29] and flow cytometry[30] for p53 protein were carried out as previously described. Equal loading of total protein was confirmed by Ponceau S staining. For pHook-selected cells, lysates were normalized for β-galactosidase activity. The stability of p53 protein in transfected cells was determined by radioactive pulse labelling of cells for 1 h and chase in unlabelled medium for 0, 1, 3, 6 and 12 h followed by immunoprecipitation of p53 using monoclonal antibody PAb421. Quantification of labelled p53 protein was carried out using the STORM phosphoimager 860 (Molecular Dynamics).

Northern blot analysis. For northern blotting, full-length p53 cDNA was used as p53 probe; the β-actin probe was obtained from Clontech.

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1. Bates, S. & Vousden, K. H. p53 in signalling checkpoint arrest or apoptosis. *Current Opin. Genet. Dev.* **6,** 1–7 (1996).
2. Barak, Y. & Oren, M. Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest. *EMBO J.* **11,** 2115–2121 (1992).
3. Momand, J., Zambetti, G. P., George, D. L. & Levine, A. J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69,** 1237–1245 (1992).
4. Chen, J. D., Marechal, V. & Levine, A. J. Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell. Biol.* **13,** 4107–4114 (1993).
5. Haupt, Y., Barak, Y. & Oren, M. Cell type-specific inhibition of p53-mediated apoptosis by mdm2. *EMBO J.* **15,** 1596–1606 (1996).
6. Chen, J., Wu, X., Lin, J. & Levine, A. J. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. *Mol. Cell. Biol.* **16,** 2445–2452 (1996).
7. Barak, Y., Juven, T., Haffner, R. & Oren, M. mdm-2 expression is induced by wild type p53 activity. *EMBO J.* **12,** 461–468 (1993).
8. Wu, X. W., Bayle, J. H., Olson, D. & Levine, A. J. The p53 mdm-2 autoregulatory feedback loop. *Genes Dev.* **7,** 1126–1132 (1993).
9. Montes de Oca Luna, R., Wagner, D. S. & Lozano, G. Rescue of early embryonic lethality in *mdm2*-deficient mice by absence of p53. *Nature* **378,** 203–206 (1995).
10. Jones, S. N., Roe, A. E., Donehower, L. A. & Bradley, A. Rescue of embyonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378,** 206–208 (1995).
11. Xiao, Z.-X. *et al.* Interaction between the retinoblasmoma protein and the oncoprotein MDM2. *Nature* **375,** 694–697 (1995).
12. Martin, K. *et al.* Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature* **375,** 691–694 (1995).
13. Marston, N. J., Crook, T. & Vousden, K. H. Interaction of p53 with MDM2 is independent of E6 and does not mediate wild type transformation suppressor function. *Oncogene* **9,** 2707–2716 (1994).
14. Lin, J., Chen, J., Elenbaas, B. & Levine, A. J. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.* **8,** 1235–1246 (1994).
15. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E. & Anderson, C. W. Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* **12,** 5041–5049 (1992).
16. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. & Howley, P. M. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63,** 1129–1136 (1990).
17. Hubbert, N. L., Sedman, S. A. & Schiller, J. T. Human papillomavirus type 16 E6 increases the degradatin rate of p53 in human keratinocytes. *J. Virol.* **66,** 6237–6241 (1992).
18. Jones, S. N. *et al.* The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. *Proc. Natl Acad. Sci. USA* **93,** 14106–14111 (1996).
19. Wrede, D., Tidy, J. A., Crook, T., Lane, D. & Vousden, K. H. Expression of RB and p53 proteins in HPV-positive and HPV-negative cervical carcinoma cell lines. *Mol. Carcinog.* **4,** 171–175 (1991).
20. Maki, C. G., Huibregtse, J. & Howley, P. M. *In vivo* ubiquitination and proteosome-mediated degradation of p53. *Cancer Res.* **56,** 2649–2654 (1996).
21. Kubbutat, M. H. G. & Vousden, K. H. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* **17,** 460–468 (1997).
22. Fenteany, G. *et al.* Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacytin. *Science* **268,** 726–731 (1995).
23. Oliner, J. D. *et al.* Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* **362,** 857–860 (1993).
24. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. *Nature* **387,** 296–299 (1997).
25. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* **8,** 2540–2551 (1994).
26. Perry, M. E., Piette, J., Zawadzki, J. A., Harvey, D. & Levine, A. J. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *Proc. Natl Acad. Sci. USA* **90,** 11623–11627 (1993).
27. Chen, C. Y. *et al.* Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. *Proc. Natl Acad. Sci. USA* **91,** 2684–2688 (1994).
28. Marston, N. J. *Mutational Analysis of the Tumour Suppressor Protein p53.* Thesis, Univ. London (1996).
29. Marston, N. J., Jenkins, J. R. & Vousden, K. H. Oligomerisation of full length p53 contributes to the interaction with mdm2 but not HPV E6. *Oncogene* **10,** 1707–1715 (1995).
30. Rowan, S. *et al.* Specific loss of apoptotic but not cell cycle arrest function in a human tumour derived p53 mutant. *EMBO J.* **15,** 827–838 (1996).

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Regulation of serotonin-2C receptor G-protein coupling by RNA editing

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The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) elicits a wide array of physiological effects by binding to several receptor subtypes. The 5-HT_2 family of receptors belongs to a large group of seven-transmembrane-spanning G-protein-coupled receptors and includes three receptor subtypes (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) which are linked to phospholipase C, promoting the hydrolysis of membrane phospholipids and a subsequent increase in the intracellular levels of inositol phosphates and diacylglycerol[1]. Here we show that transcripts encoding the 2C subtype of serotonin receptor (5-HT_{2C}R) undergo RNA editing events in which genomically encoded adenosine residues are converted to inosines by the action of double-stranded RNA adenosine deaminase(s). Sequence analysis of complementary DNA isolates from dissected brain regions have indicated the tissue-specific expression of seven major 5-HT_{2C} receptor isoforms encoded by eleven distinct RNA species. Editing of 5-HT_{2C}R messenger RNAs alters the amino-acid coding potential of the predicted second intracellular loop of the receptor and can lead to a 10–15-fold reduction in the efficacy of the interaction between receptors and their G proteins. These observations indicate that RNA editing is a new mechanism for regulating serotonergic signal transduction and suggest that this post-transcriptional modification may be critical for modulating the different cellular functions that are mediated by other members of the G-protein-coupled receptor superfamily.