Hdm2 recruits a hypoxia-sensitive co-repressor to negatively regulate p53-dependent transcription

Alexander H. Mirnezami[*], Sandra J. Campbell[†], Matthew Darley[*], John N. Primrose[*], Peter W.M. Johnson[*] and Jeremy P. Blaydes[*]

[*]Cancer Sciences Division,
School of Medicine, University of Southampton,
MP 824, Southampton General Hospital,
Southampton SO16 6YD, U.K.

†Present address: Molecular Neuropathology Laboratory,
School of Biological Sciences, University of Southampton,
Biomedical Sciences Building,
Southampton SO16 7PX, U.K.

Correspondence should be addressed to [JPB] email: j.p.blaydes@soton.ac.uk

TEL: 44-(0)23 8079 4582

FAX: 44-(0)23 8079 5152

Running title: Hdm2 recruits hCtBP2 to p53

The transcription factor p53 lies at the centre of a protein network which controls cell cycle progression and commitment to apoptosis [1]. p53 is inactive in proliferating cells, due largely to negative-regulation by the hdm2 / mdm2 oncoprotein, with which it physically associates. Release from this negative regulation is sufficient to activate p53 [2], and can be triggered in cells by multiple stimuli, through diverse pathways [3-5]. This diversity is achieved in part because hdm2 uses multiple mechanisms to inactivate p53: it targets p53 for ubiquitination and degradation by the proteosome [6-8], shuttles it out of the nucleus and into the cytoplasm [9, 10], prevents its interaction with transcriptional co-activators [11], and contains an intrinsic transcriptional repressor activity [12]. Here we show that hdm2 can also repress p53 activity through the recruitment of a known transcriptional co-repressor, hCtBP2 [13, 14]. This interaction, and consequent repression of p53-dependent transcription, is relieved under hypoxia or hypoxia-mimicking conditions that are known to increase levels of intracellular NADH. CtBP proteins can undergo an NADH-induced conformational change [15], which we show here results in a loss of their hdm2 binding ability. This pathway represents a novel mechanism whereby p53 activity can be induced by cellular stress.

RESULTS AND DISCUSSION

Screening of a human HeLa cell cDNA expression library with recombinant hdm2 protein identified CtBP2 as a potential hdm2 binding partner (not shown). A GST-human CtBP2 (GST-hCtBP2) fusion protein, but not other GST-fusion controls, was able to capture the 90 kD hdm2 protein from a partially purified bacterial lysate (Figure 1A). Furthermore, when lysates of *E. coli* which had been induced to express human proteins (either p53 or hdm2) were subjected to farwestern analysis using GST-hCtBP2 as a probe, a 90 kD band was detected only in hdm2 containing lysates (see supplementary material). These experiments demonstrate that hdm2 and hCtBP2 can associate specifically *in vitro*. Further experiments *in vitro* determined that the interaction occurs rapidly (30 min. co-incubation is sufficient for near-maximal binding under these assay conditions) and, once formed, is stable over a 24 hour incubation period and cannot be disrupted by high ionic strength (600 mM NaCl) buffer (not shown).

We then sought to determine whether the hdm2:hCtBP2 interaction can be detected *in vivo*. Hdm2 and either myc epitope-tagged hCtBP2 or myc-β-galactosidase control were transiently over-expressed in HEK 293 cells and cell lysates subjected to immunoprecipitation with anti-myc antibody. Hdm2 protein was co-immunoprecipitated from the myc-hCtBP2 containing lysates but not the myc-β-galactosidase control (see supplementary material). Similar co-immunoprecipitation experiments were performed on non-transfected MCF-7 cell lysates using anti-CtBP antibody. Hdm2 co-immunoprecipitated specifically with the anti-CtBP antibody, but not in control precipitations (Figure 1B), demonstrating a naturally-occurring interaction between endogenous hdm2 and hCtBP2 proteins *in vivo*.

hCtBP2 is a 445 a.a. protein which shares 83% sequence similarity with its homologue, hCtBP1. Both proteins have orthologues in the mouse, whereas xenopus and drosophila each express a single CtBP molecule [13, 14]. All CtBP proteins contain a highly conserved central domain with significant sequence similarity to a family of D-isomer specific 2-hydroxyacid dehydrogenases [13, 14]. This domain is located between a.a. 106-353 in hCtBP2. The N-terminus of CtBP family members contains a protein:protein interaction domain, through which they are recruited to proteins containing a consensus PXDLS motif [16, 17]. We used GST 'pull-down' assays to test a series of GST-hCtBP2 deletion mutants for their ability to bind hdm2

(Figure 1C). A mutant containing amino acids 1-110 pulled down hdm2 as effectively as the full-length protein (lane 5), whereas 110-445 did not pull-down hdm2 (lane 3). Therefore the N-terminal domain of hCtBP2 is both necessary and sufficient for the interaction with hdm2. A mutant lacking the C-terminal 86 amino acids (construct 1-359, lane 2) consistently pulled-down less hdm2 than the full length protein, despite the lack of any direct hdm2 binding-site in this region (see lane 3). This latter finding is consistent with previous reports which have indicated a regulatory role for the C-terminus of CtBP-proteins on N-terminal protein:protein interactions [18, 19].

To determine which domain of hdm2 interacts with hCtBP2, we also constructed a panel of hdm2 truncation mutants. Hdm2(6-306) bound the N-terminal domain of hCTBP2 (Figure 1D, lane 3), albeit less strongly than full length hdm2, whereas hdm2(6-210) did not bind (lane 2). The a.a. 210-306 section of hdm2 that these experiments identify as being required for hCtBP2 binding primarily consists of the acidic domain, which is known to both regulate hdm2 function [20] and interact with proteins including p300, Rb, TBP, ARF, and MTBP [21]. The hdm2 paralogue, hdmX [22], shares some homology with hdm2 within the acidic domain and can also interact with hCtBP2 *in vitro* (see supplementary material). Furthermore, the hCtBP2 paralogue, hCtBP1, is able to interact with hdm2 in GST 'pull-down' assays (see supplementary material).

The recruitment of hCtBP1 by proteins containing a PXDLS motif is regulated by changes in cellular redox potential [15]. The central dehydrogenase domain of hCtBP1 contains a high affinity binding-site for NADH (GXGXXG, Figure 2A), occupation of which induces a conformational change in the hCtBP1 molecule and an increase in binding to proteins such as E1A and ZEB. A mutation in hCtBP1 in the GXGXXG motif (G183A) abolishes NADH-responsiveness [15]. We noted that this site in hCtBP2 is conserved (a.a. 187-192) and asked whether NADH could regulate the hdm2:hCtBP2 interaction. Concentrations of NADH (0.01 to 1 mM) known to promote the interaction of hCtBP1 with PXDLS motif-proteins, inhibited binding of full length GST-hCtBP2 to hdm2 (Figure 2B, top panel). This inhibition did not occur when either GST-hCtBP2(1-110), lacking the dehydrogenase domain, or hCtBP2(G189A), containing a mutation in the NADH-binding site, were used in the assays (Figure 2B, lower panels). Therefore, in contrast to interactions with PXDLS-motif proteins, the conformational changes induced by NADH-binding to the CtBP dehydrogenase domain result in a reduced

affinity of hCtBP2 for hdm2. Exposure of cells in culture to $CoCl_2$ can be used as a model for the induction of a hypoxia-like stress-response. Zhang *et* al [15] have shown that $CoCl_2$ treatment (200μM) induces an increase in the cellular NADH/NAD^+ ratio sufficient to promote binding of CtBP proteins to PXDLS motif-proteins in the cell. As shown in Figure 2C, 200 μM $CoCl_2$ reduced the formation of hdm2:hCtBP2 complexes in MCF-7 cells. Hypoxia, which has a greater effect on the cellular NADH/NAD^+ ratio than $CoCl_2$ [15], was more effective than $CoCl_2$ in reducing the hdm2:hCtBP2 interaction (Figure 2C). These data demonstrate, therefore, that the NADH-induced regulation of the hdm2:hCtBP2 interaction also occurs *in vivo.*

To determine the functional consequences of the hdm2:hCtBP2 interaction, we examined whether over-expression of either molecule would modify the function of the other. Hdm2 regulates p53 levels in cells by directing the nuclear export of p53 and targeting it to the ubiquitin-proteasome degradation pathway [6-10]. We therefore tested whether hdm2 could regulate hCtBP2 protein levels. HEK 293 cells were transiently transfected with vectors encoding myc-tagged hCtBP2, and increasing amounts of hdm2. Hdm2 did not decrease hCtBP2 expression levels in this assay (see supplementary material) providing evidence that hdm2 does not target hCtBP2 for proteosome-mediated degradradation, nor did it have an effect on the intracellular localisation of hCtBP2 in HEK 293 cells (data not shown). The best documented function of CtBP proteins is as short-range transcriptional co-repressors [13, 14]. Thus, CtBP proteins are recruited to promoters by sequence specific DNA-binding transcription factors, either through a direct physical interaction, or indirectly through bridging proteins. We therefore tested whether the formation of an hdm2:hCtBP2 complex could modify the ability of hdm2 to repress p53-dependent transcription. For this experiment we used three different p53 responsive luciferase reporters: bax-Luc, hdm2-Luc03, and p21-Luc containing promoter regions from bax, hdm2 and $p21^{WAF-1}$ respectively. In the presence of hdm2, hCtBP2 reproducibly repressed p53-dependent transcription from the bax-Luc and hdm2-Luc reporters, but was without effect on p21-Luc (Figure 3A).

To confirm that the inhibition of p53-dependent transcription by hCtBP2 is dependent on a direct interaction between hdm2 and hCtBP2, cells were treated with $CoCl_2$ to disrupt the hdm2:hCtBP2 interaction. Following $CoCl_2$ exposure, hCtBP2 was no longer able to enhance hdm2-mediated repression of p53-dependent transcription (Figure 3B), this was not due to an

effect on p53 protein levels in these cells (data not shown). This effect of $CoCl_2$ was due to its specific effect on regulating intracellular NADH levels, since repression by the hCtBP2(G189A) mutant was unaffected by $CoCl_2$ (Figure 3C). In addition, we transfected cells with an hdm2(1-210) mutant which can bind p53, is localised in the nucleus, and can inhibit p53 activity, but cannot bind hCtBP2. In the presence of hdm2(1-210), hCtBP2 did not repress p53-dependent transcription. (Figure 3D), confirming that an interaction between hdm2 and hCtBP2 is required for hCtBP2 to be able to repress p53-dependent transcription.

One model that could account for our observation is that an hdm2:hCtBP2 complex directly interacts with p53 to bring about repression of p53 transactivating ability. The binding sites for p53 and hCtBP2 on hdm2 are located in distinct domains, suggesting that a trimeric complex of p53:hdm2:hCtBP2 could form. Figure 4A demonstrates that, when combinations of hdm2, GST-hCtBP2(1-110), and p53 are co-incubated *in vitro*, p53 co-precipitates with GST-hCtBP2 (1-110) only in the presence of hdm2. Therefore hdm2 is able to recruit hCtBP2 to p53. In this model (Figure 4B) the role of hdm2 as a bridging protein between hCtBP2 and p53 is analogous to the role played by CtIP in recruiting CtBP to BRCA1 [23]. Our model is further supported by recent evidence that hdm2 can be recruited to p53-responsive promoters through p53 binding [24]. The promoter specificity of this effect in our assays may reflect the known requirement for CtBP proteins to be recruited to sites located within a short distance of the promoter for repression to occur [17]. Consistent with this known property of CtBP proteins, the p53-responsive promoters used in this study which were sensitive to inhibition by hCtBP2 (bax-Luc and hdm2-Luc03) both have p53-response elements (p53-RE) situated within 500 b.p. of the transcription start site, whereas in the non-responsive p21-promoter, the p53-RE is located further upstream at –2400. It is important to consider, however, that the mechanisms whereby CtBP represses transcription remain largely undefined, and may be dependent on the context of individual promoters. Specifically, CtBP proteins can recruit histone deacetylase proteins, including HDAC1 [25, 26], and repression by CtBP can be either depdendent [16] or independent [27] of HDAC activity. Hdm2 has recently be shown to recruit HDAC1 to p53 though an undefined bridging protein, resulting in de-acetylation of p53 and a reduction in the expression of p53-response genes including hdm2 and $p21^{WAF-1}$ [28]. If CtBP proteins function as this bridging molecule, then they could be predicted to have HDAC1-dependent inhibitory effects on promoters such as $p21^{WAF1}$,

in addition to the effects on the hdm2 and bax promoters which were observed in the absence of HDAC1 transfection.

Whilst we have clearly demonstrated that a hypoxia-regulated interaction occurs between endogenous hdm2 and hCtBP2 proteins, our data defining the functional consequence of this interaction is dependent of the analysis of synthetic reporter constructs. However, recent analysis of gene expression profiles in mouse embryo fibroblasts (MEFs) from CtBP1 and 2 homozygous null animals has recently confirmed that CtBP can repress expression of a range of endogenous p53 target genes, including Bax, PERP and Noxa [29]. Expression of all of these genes was up-regulated in CtBP null MEFs, and re-repressed when CtBP2 was re-expressed in these cells. Reporter-based assays performed by the authors of this manuscript lead them to conclude that this effect was independent of a direct effect of CtBP on p53, and the mechanism underlying the inhibition was not defined. However their assays were performed in the absence of hdm2 co-transfection, and under conditions of chemotherapeutic drug exposure, which would have disrupted the interaction between hdm2 and p53, and therefore would have been unlikely to have detected the effects that we have demonstrated here. The authors also clearly demonstrated that CtBP null MEFs are hypersensitive to cell killing by a range of pro-apoptotic stimuli, with clear implications for the role of CtBP family proteins in tumour biology. Evidence that we have presented here provides, in part, a mechanistic explanation for these striking observations.

Hdm2 is emerging as a key regulator of cell-cycle control and commitment to apoptosis [21]. The identification of protein:protein interactions made by hdm2 has resulted in a number of significant advances in our understanding of how these processes can be controlled by diverse cellular stimuli, such as the activation of oncogenes [30] and ionising radiation [4]. Several hdm2-binding proteins are also promising targets for the design of novel cancer therapies [31]. Hypoxic stress induces a p53-dependent apoptotic response, and in many types of cancer may be responsible for the positive growth advantage of tumour cells in which the p53 gene is mutated [32]. The mechanisms whereby hypoxia, and hypoxia-mimicking chemicals such as $CoCl_2$ and deferoxamine, induce p53-dependent apoptosis are now beginning to be elucidated. There is evidence that the mechanism may either be dependent, or independent, on the activation of p53's trans-activation activity, depending on the cell type being studied, and the exact nature of the stress involved [5, 33]. Our finding that hypoxia-mimicking conditions result in the dissociation

of hdm2:hCtBP2 complexes, and a de-repression of p53-dependent transcription, provides a significant advance in the understanding of these pathways.

CtBP family members interact with, and modify the activity of, a large number of proteins with key roles in development, differentiation, cell-cycle control and apoptosis [13, 14, 34]. The majority of the interactions that have been described to date are dependent on the presence of a PXDLS motif in the interacting protein. In a recent two-hybrid analysis [35], however, 13 of 41 CtBP-interacting clones did not contain this motif, and similarly, there is no PXDLS sequence in hdm2. We have demonstrated that the NADH-induced conformational change in CtBP family members, which is known to result in an increase in their affinity for PXDLS motif-proteins, causes a decrease in hCtBP2 binding to hdm2 protein, and we anticipate the discovery of other CtBP-protein interactions which are regulated by NADH in this way. In summary, our data demonstrates that NADH-binding by CtBP proteins does not act as a simple 'on-off switch' for binding to one set of transcription factors, but rather a mechanism to regulate the differential recruitment of CtBP by two distinct groups of proteins in the cell.

Supplementary material

Supplementary material including additional methodological details is available on the current biology website

ACKNOWLEDGMENTS

AHM is a Medical Research Council clinical training fellow. Funding for antibody production was from the Peel Medical Research Trust. We are grateful to A.J.Levine, T.Elliott, G.Packham. R.Grand and M.Oren for antibody and plasmid reagents, Dr. L.Burch for hdm2 protein for the library screen, and Dr. T.R.Hupp for encouragement in the initial stages of the study. The manuscript was critically appraised by Professor T.Elliott.

REFERENCES

1. Vogelstein B, Lane D, Levine AJ: **Surfing the p53 network**. *Nature* 2000, **408**:307-310.

2. Blaydes JP, Wynford-Thomas D: **The proliferation of normal human fibroblasts is dependent upon negative regulation of p53 function by mdm2**. *Oncogene* 1998, **16**:3317-3322.

3. Prives C: **Signaling to p53: breaking the MDM2-p53 circuit**. *Cell* 1998, **95**:5-8.

4. Wahl GM, Carr AM: **The evolution of diverse biological responses to DNA damage: insights from yeast and p53**. *Nat Cell Biol* 2001, **3**:E277-286.

5. Ashcroft M, Taya Y, Vousden KH: **Stress signals utilize multiple pathways To stabilize p53**. *Mol Cell Biol* 2000, **20**:3224-3233.

6. Haupt Y, Maya R, Kazaz A, Oren M: **Mdm2 promotes the rapid degradation of p53**. *Nature* 1997, **387**:296-299.

7. Kubbutat MH, Jones SN, Vousden KH: **Regulation of p53 stability by Mdm2**. *Nature* 1997, **387**:299-303.

8. Honda R, Tanaka H, Yasuda H: **Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53**. *FEBS Lett* 1997, **420**:25-27.

9. Roth J, Dobbelstein M, Freedman DA, Shenk T, Levine AJ: **Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein**. *EMBO J.* 1998, **17**:554-564.

10. Freedman DA, Levine AJ: **Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6**. *Mol Cell Biol* 1998, **18**:7288-7293.

11. Wadgaonkar R, Collins T: **Murine double minute (MDM2) blocks p53-coactivator interaction, a new mechanism for inhibition of p53-dependent gene expression**. *J Biol Chem* 1999, **274**:13760-13767.

12. Thut CJ, Goodrich JA, Tjian R: **Repression of p53-mediated transcription by MDM2: a dual mechanism**. *Genes Dev* 1997, **11**:1974-1986.

13. Chinnadurai G: **CtBP, an unconventional transcriptional corepressor in development and oncogenesis**. *Mol Cell* 2002, **9**:213-224.

14. Turner J, Crossley M: **The CtBP family: enigmatic and enzymatic transcriptional co-repressors**. *Bioessays* 2001, **23**:683-690.

15. Zhang Q, Piston DW, Goodman RH: **Regulation of corepressor function by nuclear NADH**. *Science* 2002, **295**:1895-1897.

16. Criqui-Filipe P, Ducret C, Maira SM, Wasylyk B: **Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation**. *Embo J* 1999, **18**:3392-3403.

17. Koipally J, Georgopoulos K: **Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity**. *J Biol Chem* 2000, **275**:19594-19602.

18. Furusawa T, Moribe H, Kondoh H, Higashi Y: **Identification of CtBP1 and CtBP2 as corepressors of zinc finger- homeodomain factor deltaEF1**. *Mol Cell Biol* 1999, **19**:8581-8590.

19. Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H: **The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling**. *Blood* 2001, **97**:2815-2822.

20. Argentini M, Barboule N, Wasylyk B: **The contribution of the acidic domain of MDM2 to p53 and MDM2 stability**. *Oncogene* 2001, **20**:1267-1275.

21. Daujat S, Neel H, Piette J: **MDM2: life without p53**. *Trends Genet* 2001, **17**:459-464.

22. Shvarts A, Bazuine M, Dekker P, Ramos YF, Steegenga WT, Merckx G, van Ham RC, van der Houven van Oordt W, van der Eb AJ, Jochemsen AG: **Isolation and identification of the human homolog of a new p53-binding protein, Mdmx**. *Genomics* 1997, **43**:34-42.

23. Li S, Chen PL, Subramanian T, Chinnadurai G, Tomlinson G, Osborne CK, Sharp ZD, Lee WH: **Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage**. *J Biol Chem* 1999, **274**:11334-11338.

24. Jin Y, Zeng SX, Dai MS, Yang XJ, Lu H: **MDM2 Inhibits PCAF-mediated p53 acetylation**. *J Biol Chem* 2002, **14**:14.

25. Sundqvist A, Sollerbrant K, Svensson C: **The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex**. *FEBS Lett* 1998, **429**:183-188.

26. Subramanian T, Chinnadurai G: **Association of class I histone deacetylases with transcriptional corepressor CtBP**. *FEBS Lett* 2003, **540**:255-258.

27. Meloni AR, Smith EJ, Nevins JR: **A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor**. *Proc Natl Acad Sci U S A* 1999, **96**:9574-9579.

28. Ito A, Kawaguchi Y, Lai CH, Kovacs JJ, Higashimoto Y, Appella E, Yao TP: **MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation**. *Embo J* 2002, **21**:6236-6245.

29. Grooteclaes M, Deveraux Q, Hildebrand J, Zhang Q, Goodman RH, Frisch SM: **C-terminal-binding protein corepresses epithelial and proapoptotic gene expression programs**. *Proc Natl Acad Sci U S A* 2003, **3**:3.

30. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G: **The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2**. *EMBO J.* 1998, **17**:5001-5014.

31. Midgley CA, Desterro JM, Saville MK, Howard S, Sparks A, Hay RT, Lane DP: **An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo**. *Oncogene* 2000, **19**:2312-2323.

32. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ: **Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours**. *Nature* 1996, **379**:88-91.

33. Koumenis C, Alarcon R, Hammond E, Sutphin P, Hoffman W, Murphy M, Derr J, Taya Y, Lowe SW, Kastan M, Giaccia A: **Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation**. *Mol Cell Biol* 2001, **21**:1297-1310.

34. Hildebrand JD, Soriano P: **Overlapping and unique roles for C-terminal binding protein 1 (CtBP1) and CtBP2 during mouse development**. *Mol Cell Biol* 2002, **22**:5296-5307.

35. Vo N, Fjeld C, Goodman RH: **Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP**. *Mol Cell Biol* 2001, **21**:6181-6188.

FIGURE LEGENDS

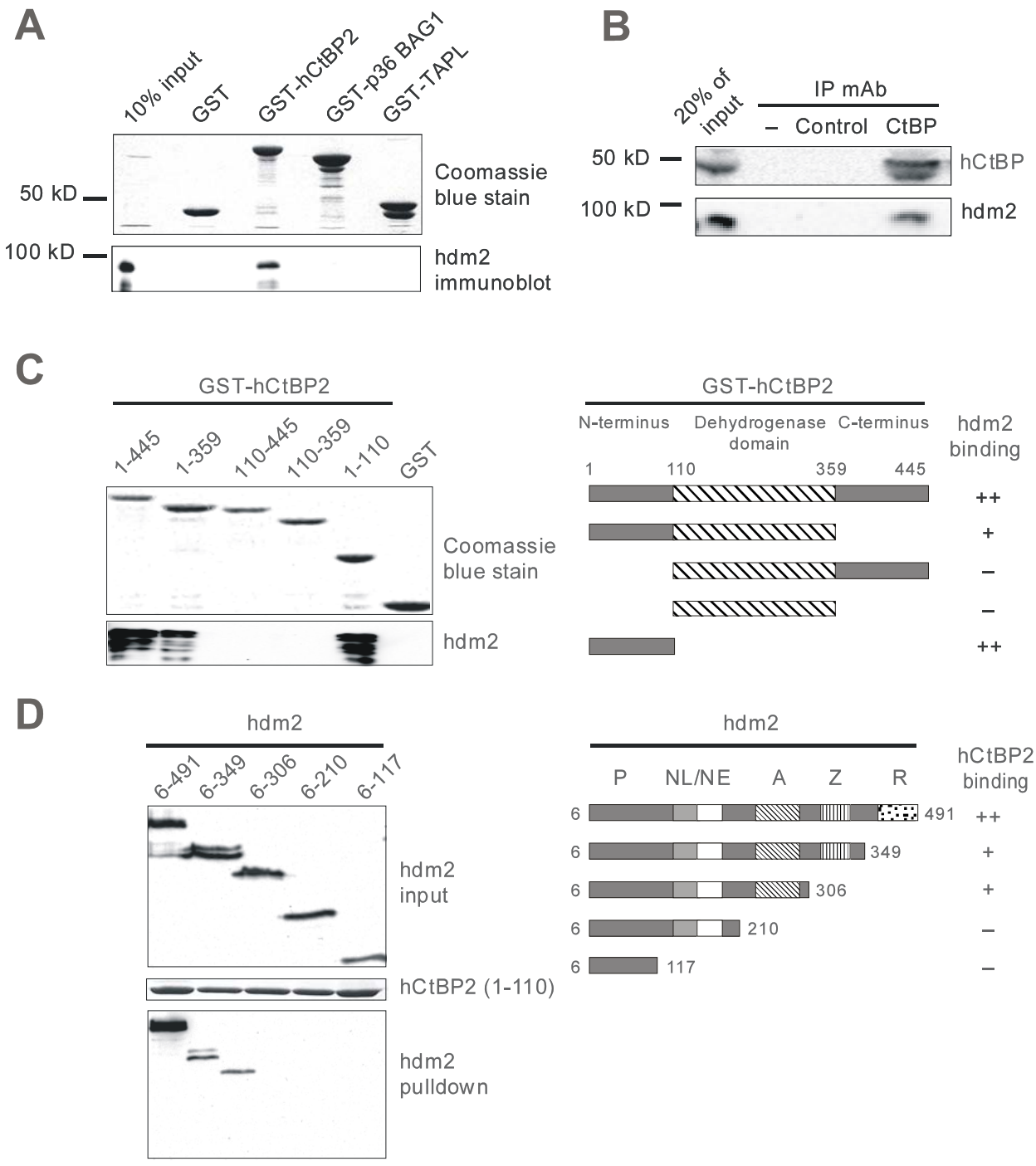
Figure 1 Hdm2 interacts with hCtBP2 *in vitro* and *in vivo***.** (A) Partially purified recombinant hdm2 was incubated with the GST-fusion proteins indicated, and complexes pulled down using glutathione-sepharose beads. Samples were then analysed by SDS-PAGE followed by either Coomassie blue staining to demonstrate equal amounts of GST-fusion protein, or western blotting with mAb 2A10 to detect hdm2. (B) Extracts from MCF-7 cells were immunoprecipitated with protein G beads alone (-), an irrelevant goat Ig to caspase 6 (control), or E16 goat-polyclonal antibody to CtBP. Precipitates were then analysed by immunoblotting, using rabbit polyclonal Ig R41 to hCtBP2, and hdm2 mAb 2A9. (C) Recombinant full length GST-hCtBP2(1-445), GST-hCtBP2 deletion mutants, and GST alone, were used as bait in GST 'pull-down' assays with equal amounts of full length hdm2. Coomassie blue staining was used to confirm equivalent amounts of the CtBP2 mutants had been used (upper panel) and immunoblotting with mAb 2A10 was used to detect co-immunoprecipitated hdm2 (lower panel). A comparison between the mutants and the known domains of hCtBP2 is shown, with the N-terminal GST-fusion omitted. (D) GST-hCtBP2(1-110) was used to pull down the hdm2 deletion mutants indicated. Equal amounts of input proteins were confirmed using western blotting with mAb 4B2 for hdm2 and Coomassie blue staining for GST-hCtBP2(1-110). Co-immunoprecipitated hdm2 was detected by immunoblotting with mAb 4B2. The location of the hdm2 deletion mutants with reference to known domains of hdm2 are shown: (P)=p53-binding domain; (NL/NE)=Nuclear localisation and export sequences; (A)=acidic region; (Z)=Zinc finger; (R)=RING finger.

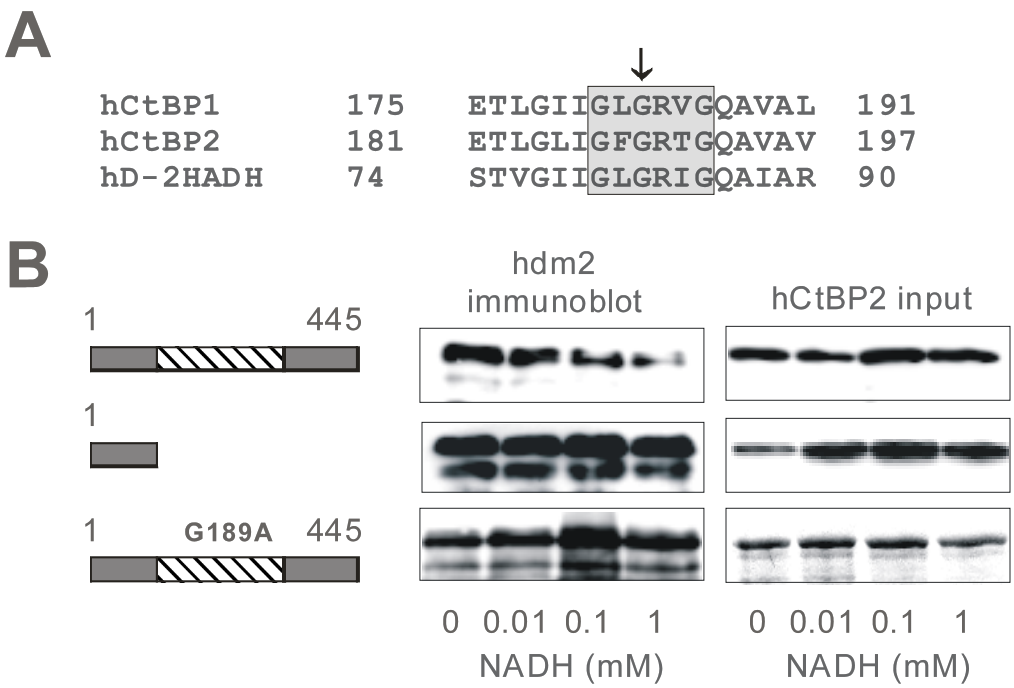
Figure 2 The hdm2:hCtBP2 interaction is inhibited by NADH-binding to the hCtBP2 dehydrogenase domain. (A) Multiple sequence alignment of the conserved GXGXXG NAD^+/NADH binding site of hCtBP1, hCtBP2, and the human D-isomer specific 2-Hydroxyacid Dehydrogenase enzyme (hD-2HADH). The affinity of the site in hCtBP1 has been reported to be approximately 100 fold greater for NADH than NAD^+ [15]. The arrow marks the site of the alanine substitution mutant in hCtBP2 (G189A). (B) The interaction between hdm2 and the indicated GST-hCtBP2 fusion proteins was determined by GST 'pull-down' assay in the presence of increasing concentrations of NADH. (C) MCF-7 cells were treated for 2 hr with 200 μM $CoCl_2$ or hypoxia (<1% O_2) where indicated. Cell lysates were immunoprecipitated (IP) with

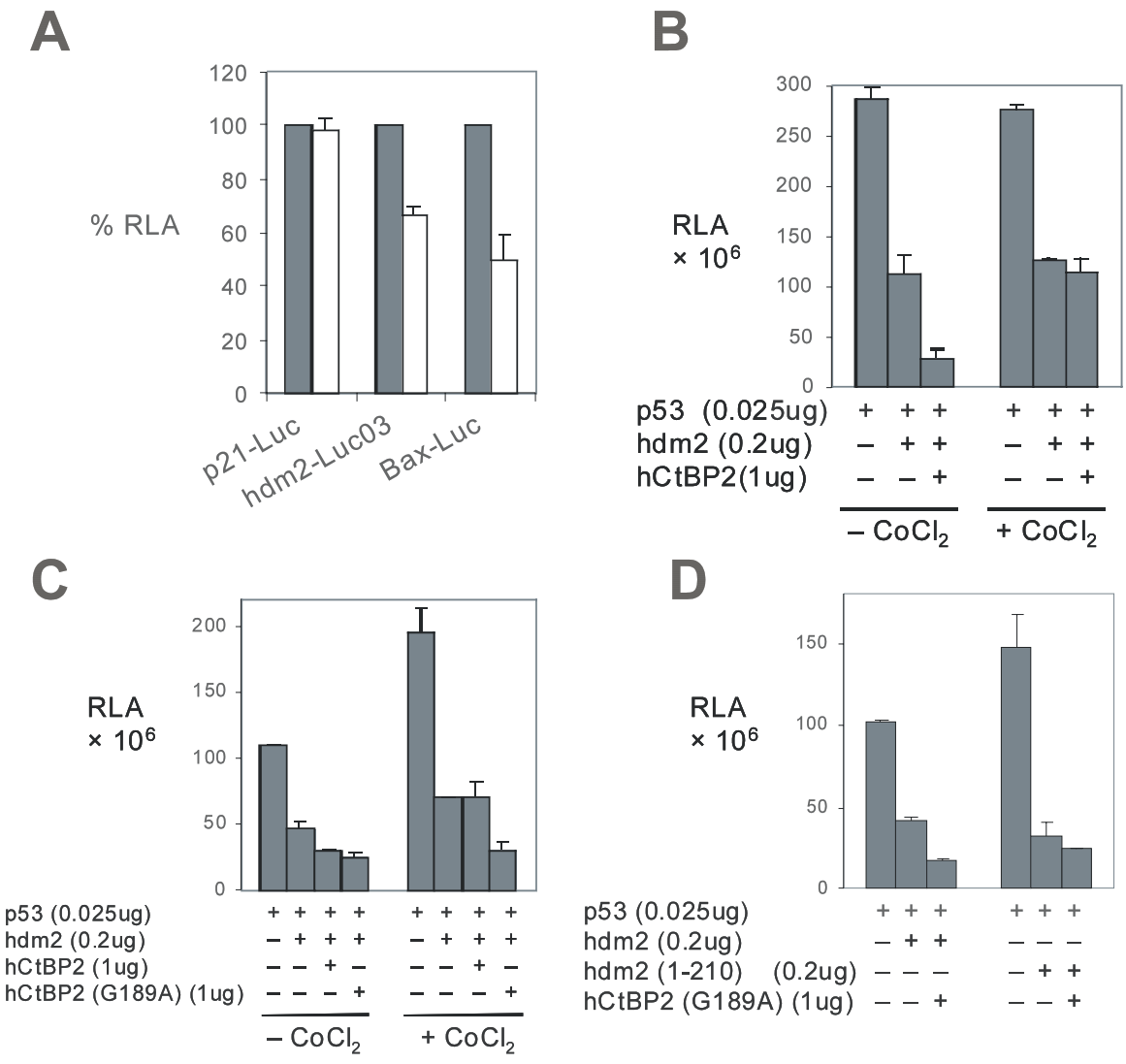
goat polyclonal anti-CTBP (E-16), or a control antibody to caspase 6 and immunoprecipitated proteins analysed by western blot with antibodies for CtBP (R41) and hdm2 (2A10).

Figure 3 Recruitment of hCtBP2 by hdm2 results in promoter-selective repression of p53-dependent transcription. (A) HEK 293 cells were transfected with 200 ng of the p53-dependent reporter vector containing either $p21^{WAF1}$, hdm2 or bax promoter sequences, 25 ng of wild-type p53 expression vector, and 200 ng of hdm2 expression vector to give conditions where approximately 70% inhibition of p53 activity by the co-transfected hdm2 was achieved. Transfections also included 1 μg of either pcDNA 3.1 (solid bars) or pcDNA3mychis hCTBP2 (open bars) expression vectors. Relative luciferase activity (RLA) was first calculated by normalisation to expression from a co-transfected β-galactosidase expression vector, and the RLA of each promoter in the presence of pcDNA3.1 was defined as 100%. In the absence of p53, promoter activity was approximately 2%. (B) HEK 293 cells were transfected with the hdm2-Luc03 p53-responsive reporter vector (which contains only 165 b.p. of hdm2 promoter sequence including the two p53 response elements) and the indicated amounts of expression vector. 30 hr post-transfection, cells were treated for 16 hr with 200 μM $CoCl_2$ before assaying. (C) Transfections were repeated as in (B), with the addition of the NADH-insensitive G189A mutant of hCtBP2. (D) Hdm2-Luc03 reporter assays in HEK 293 cells were performed using the indicated vectors. hCtBP2(G189A) was used in this experiment as it functions as a constitutively-active mutant which is insensitive to variations in intracellular NADH.

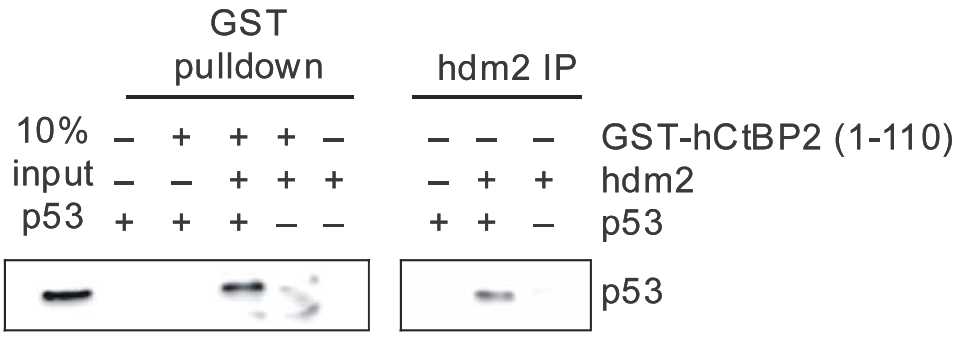
Figure 4 Proposed model of the mechanism whereby hdm2 recruits CtBP proteins to negatively regulate p53-dependent transcription. (A) Recombinant human p53, hdm2, and GST-hCtBP2(1-110) were mixed as indicated and subjected to either GST 'pull-down' analysis or immunoprecipitation with mAb 2A9 to hdm2. Co-precipitated p53 was detected with polyclonal antibody CM1. (B) Model for the promoter-selective inhibition of p53-dependent transcription by CtBP proteins. Given the high degree of functional homology between hCtBP1 and hCtBP2, and the ability of the two proteins to form hetero-dimers, it is probable that hCtBP1 is also able to repress p53 activity by this mechanism.


A
10% input
GST
GST-hCtBP2
GST-p36 BAG1
GST-TAPL
50 kD
100 kD
Coomassie blue stain
hdm2 immunoblot
B
20% of input
IP mAb
– Control CtBP
50 kD
100 kD
hCtBP
hdm2
C
GST-hCtBP2
1-445
1-359
110-445
110-359
1-110
GST
Coomassie blue stain
hdm2
GST-hCtBP2
N-terminus
Dehydrogenase domain
C-terminus
hdm2 binding
1
110
359
445
++
+
–
–
++
D
hdm2
6-491
6-349
6-306
6-210
6-117
hdm2 input
hCtBP2 (1-110)
hdm2 pulldown
hdm2
P
NL/NE
A
Z
R
hCtBP2 binding
6
491
++
6
349
+
6
306
+
6
210
–
6
117
–

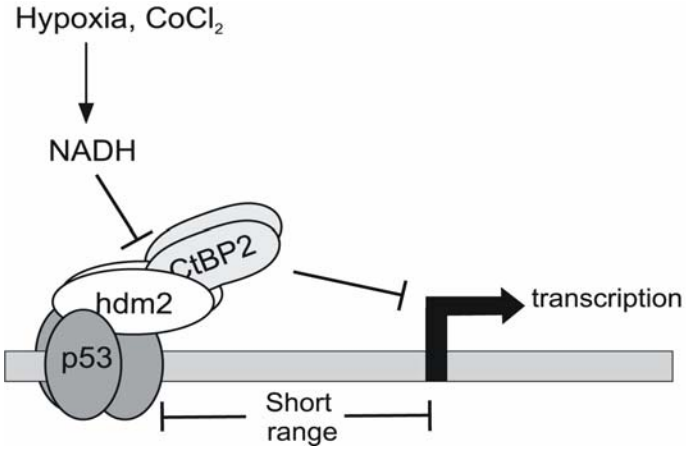

A
hCtBP1 175 ETLGIIGLGRVGQAVAL 191
hCtBP2 181 ETLGLIGFGRTGQAVAV 197
hD-2HADH 74 STVGIIGLGRIGQAIAR 90
B
1
445
1
1
G189A
445
hdm2
immunoblot
hCtBP2 input
0 0.01 0.1 1
NADH (mM)
0 0.01 0.1 1
NADH (mM)
C
Control
CoCl2
Hypoxia
Treatment
20% of input
Control
CtBP
Control
CtBP
Control
CtBP
IP
hCtBP
hdm2


A
% RLA
120
100
80
60
40
20
0
p21-Luc
hdm2-Luc03
Bax-Luc
B
RLA
× 10^6
300
250
200
150
100
50
0
p53 (0.025ug) + + + + + +
hdm2 (0.2ug) – + + – + +
hCtBP2(1ug) – – + – – +
– CoCl2
+ CoCl2
C
RLA
× 10^6
200
150
100
50
0
p53 (0.025ug) + + + + + + + +
hdm2 (0.2ug) – + + + – + + +
hCtBP2 (1ug) – – + – – – + –
hCtBP2 (G189A) (1ug) – – – + – – – +
– CoCl2
+ CoCl2
D
RLA
× 10^6
150
100
50
0
p53 (0.025ug) + + + + + +
hdm2 (0.2ug) – + + – – –
hdm2 (1-210) (0.2ug) – – – – + +
hCtBP2 (G189A) (1ug) – – + – – +

A


GST
pulldown
hdm2 IP
10% – + + + – – – – GST-hCtBP2 (1-110)
input – – + + + – + + hdm2
p53 + + + – – + + – p53
p53

B


Hypoxia, $CoCl_2$
NADH
CtBP2
hdm2
p53
transcription
Short
range

SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Library screen

A HeLa cell cDNA Uni-ZAP XR protein expression library (Stratagene) was screened according to the manufacturers instructions. Plaques were lifted onto nitrocellulose filters, blocked with 1% bovine serum albumin (BSA) in 0.05% T-TBS (0.05% Tween-20 in Tris-buffered saline) and probed with pre-formed complexes of recombinant *E. coli*-expresssed hdm2 (100 ng/ml) and mAb 4B2 (0.5 ng/ml) in 3% BSA, 1% bacterial cell lysate (Stratagene), 1 mM Benzamidine, 5 mM DTT, 0.05% T-TBS. Following extensive washing in 0.1%T-TBS, bound complexes were detected with alkaline phosphatase-conjugated rabbit anti-mouse antibody, followed by washing and detection by BCIP-NBT reagent (Stratagene). Positive plaques were subjected to further screening, before excision of the pbluescipt (pBSK) phagemid containing the cDNA.

Expression vectors, proteins, and antibodies

The full length GST-hCtBP2 expression vector and the panel of GST-hCtBP2 deletion constructs were cloned into the pGEX-2TK expression vector (Amersham Biosciences) by proof reading-PCR amplification of the pBSK-hCtBP2 plasmid which had been derived from the cDNA library screen. Full length hCtBP2 was also cloned into the pcDNA3-mychisA vector (Invitrogen) using *BamHI* and *KpnI* restriction sites. A G189A mutation was introduced into hCtBP2 using side-directed mutagenesis (Quickchange, Stratagene). pT7.7hdm2(6-491) and pCMVhdm2(1-491) vectors were used to generate hdm2 truncation mutants deletions by the insertion of TGA stop codons. Expression of soluble protein in *E.*coli from pT7.7hdm2, pT7.7hdmX, pGEX-2TK-hCtBP2 and pGEX-4T1-hCtBP1 vectors was performed using established procedures. Human p53 was expressed in *Sf9* cells and partially purified using heparin-sepharose chromatography. P21-Luc and bax-Luc reporter vectors have been described previously, hdm2-Luc03 contains 165 b.p. of the hdm2 P2 promoter including the two p53-response elements. R41 antibody to recombinant hCtBP2, and anti-hdmX were generated by Moravian Technologies.

Tissue culture, transfection and reporter assays

Human HEK 293 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Hypoxia (<1% O_2) was generated using an Anaerogen

hypoxic chamber system (Oxoid, U.K.). Transient transfections were conducted using the TransFast™ transfection reagent (Promega). For each transfection, the total amount of DNA used was kept constant by adding the relevant empty vector. pcDNA3mychis-LacZ (β-galactosidase) was included in all reporter assay transfections to allow normalisation for transfection efficiency. For reporter assays, cells were harvested 48 hours after transfection and lysed in reporter lysis buffer (0.65% IGEPAL CA-630, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM NaCl) for 2 min at room temperature before centrifuging at 13,000 g for 3 min. Supernatants were assayed for Luciferase activity using LucLite reagent (Packard), and a TopCount microplate luminometer (Packard). Luciferase activity was normalised to β-galactosidase activity in all experiments. Assays were performed in duplicate, and representative data from repeat experiments is shown.

Protein analysis

For *in vitro* binding assays, GST fusion proteins were incubated overnight at 4 ˚C with 10-20 µl of glutathione-sepharose beads slurry (Amersham Pharmacia) and subsequently washed 3-5 times with ice-cold NETS buffer (50 mM Tris.HCl pH 7.6, 150 mM NaCl, 5 mM EDTA, 1% IGEPAL CA-630, 5mM DTT), to remove unbound proteins. Samples were incubated for 1 hr with recombinant hdm2 at room temperature, before washing again. Precipitated proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE for western blotting. For immunoprecipitation from whole cell lysates**,** cells were rinsed in ice-cold PBS, scraped, and pelleted. Lysis was conducted with 1-2 volumes of NETS buffer containing Complete™ protease inhibitor (Boehringer Ingelheim) for 15 minutes on ice before centrifuging the sample at 13,000g for 10 min. Supernatants were then pre-cleared using a small volume of protein G-sepharose beads, and immunoprecipitated overnight at 4˚ C with either the mAb 9E10 to the myc tag, or the polyclonal goat antibodies E16 (CtBP1&2, Santa Cruz) and K-20 (caspase 6, Santa Cruz). Precipitated proteins were then recovered by incubating the mixture with protein G-sepharose beads for 1 hr at room temperature, fractionated by 10% SDS-PAGE, and transferred onto nitrocellulose. Proteins were detected using the mAb 2A9 and 2A10 (hdm2), 3G10 (GST, Abcam), and polyclonal antibodies E16 and R41 (hCtBP2). For farwestern analysis, bacterial lysate containing either recombinant GST-hCtBP2 or GST (in 5% milk, 0.1% Tween-20, PBS) was used to probe for hCtBP2 binding proteins. Secondary detection was with mAb 3G10.

FIGURE LEGENDS

Figure S1 Hdm2 and hCtBP2 interact *in* vitro and *in vivo* (A) Farwestern analysis of the hdm2:hCtBP2 interaction. IPTG-induced cell lysates from *E.coli* BL21 (DE3) cells that had been transformed with either no plasmid (control), pT7.7hdm2, or pT7.7p53 were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Identical membranes were either probed directly for hdm2 using mAb 2A9, or subjected to farwestern analysis using either GST-hCtBP2 or GST protein as a probe, and anti-GST antibody for detection. Gels were stained with Coomassie blue to confirm that equal amounts of bacterial lysate were loaded. (B) HEK 293 cells were transfected with pCMVhdm2 and either pcDNA3mychis-LacZ (β-galactosidase) or pcDNA3mychis-hCTBP2 expression vectors. 40 hr later, cells were treated with 20 μM MG132 (to prevent proteasomal degradation of hdm2) for 2 hr before cell lysis. Immunoprecipitation (IP) was performed with mAb 9E10 to myc, and co-immunoprecipitated hdm2 detected by mAb 2A10. Comparable levels of both hdm2 (lanes 1 and 2), and the myc-tagged transfected proteins (not shown) in the input cell-lysates were also confirmed

Figure S2 Interaction between hdm2 and hCtBP2 paralogues. (A) HdmX was subjected to GST 'pull-down' analysis using GST-hCtBP2 or GST alone as bait. Detection was with polyclonal antisera to hdmX. (B) Hdm2 was subjected to GST 'pull-down' analysis using either GST alone, GST-hCtBP2 or GST-hCtBP1 as indicated

Figure S3 Hdm2 does not target hCtBP2 for degradation. HEK 293 cells were transfected with increasing amounts of hdm2 and equal amounts of myc-hCtBP2 and myc-β-galactosidase expression vectors as indicated. 40 hr later, cell extracts were prepared, and western blotted using mAb 9E10 for myc-epitope detection, and mAb 2A10 for hdm2.

Fig S1

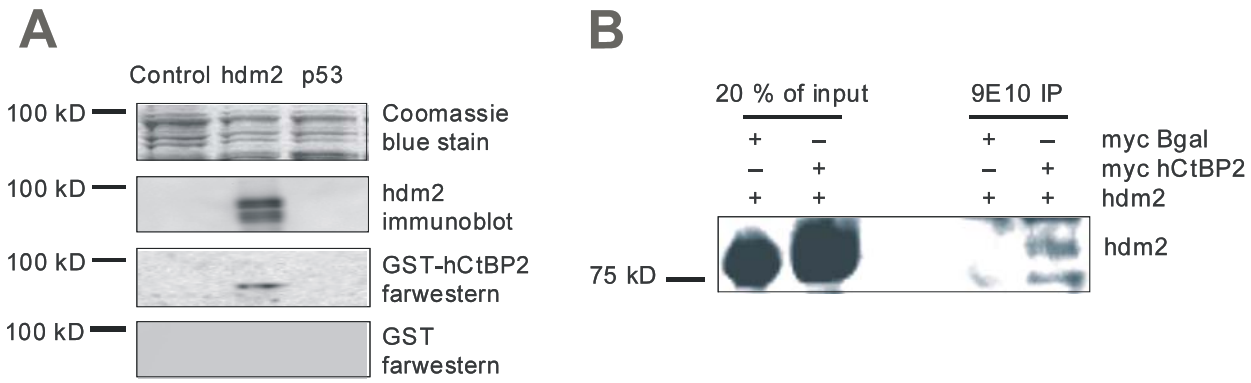


Fig S2

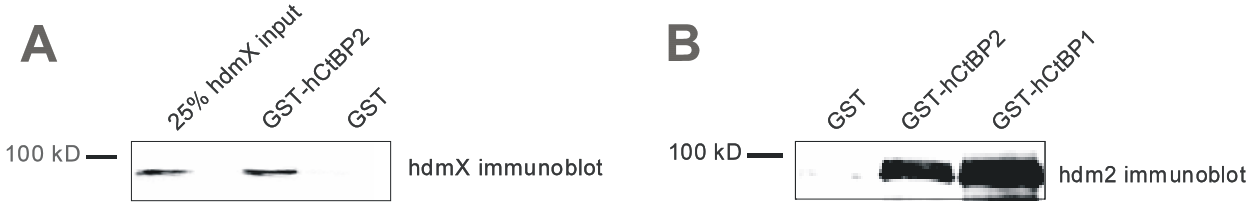


Fig S3