Functional Inactivation of Endogenous MDM2 and CHIP by HSP90 Causes Aberrant Stabilization of Mutant p53 in Human Cancer Cells

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Abstract

The tight control of wild-type p53 by mainly MDM2 in normal cells is permanently lost in tumors harboring mutant p53, which exhibit dramatic constitutive p53 hyperstabilization that far exceeds that of wild-type p53 tumors. Importantly, mutant p53 hyperstabilization is critical for oncogenic gain of function of mutant p53 in vivo. Current insight into the mechanism of this dysregulation is fragmentary and largely derived from ectopically constructed cell systems. Importantly, mutant p53 knock-in mice established that normal mutant p53 tissues have sufficient enzymatic reserves in MDM2 and other E3 ligases to maintain full control of mutant p53. We find that in human cancer cells, endogenous mutant p53, despite its ability to interact with MDM2, suffers from a profound lack of ubiquitination as the root of its degradation defect. In contrast to wild-type p53, the many mutant p53 proteins which are conformationally aberrant are engaged in complexes with the HSP90 chaperone machinery to prevent its aggregation. In contrast to wild-type p53 cancer cells, we show that in mutant p53 cancer cells, this HSP90 interaction blocks the endogenous MDM2 and CHIP (carboxy-terminus of Hsp70-interacting protein) E3 ligase activity. Interference with HSP90 either by RNA interference against HSF1, the transcriptional regulator of the HSP90 pathway, or by direct knockdown of Hsp90 protein or by pharmacologic inhibition of Hsp90 activity with 17AAG (17-allylamino-17-demethoxygeldanamycin) destroys the complex, liberates mutant p53, and reactivates endogenous MDM2 and CHIP to degrade mutant p53. Of note, 17AAG induces a stronger viability loss in mutant p53 than in wild-type p53 cancer cells. Our data support the rationale that suppression of mutant p53 levels in vivo in established cancers might achieve clinically significant effects.

Mol Cancer Res; 9(5); 577–88. ©2011 AACR.

Introduction

Missense mutations in the p53 gene occur in more than 50% of human cancers. In normal unstressed cells, the level of wild-type p53 protein is very low due to rapid turnover by its main physiologic E3 ligase MDM2, which is interrupted only when needed in response to stress. This tight control is permanently lost in tumors harboring mutant p53, which exhibits a dramatic constitutive p53 stabilization compared with wild-type p53 tumors. Mutant p53 hyperstability was always assumed to be entirely due to the loss of p53-mediated transactivation of MDM2, itself a wild-type p53 target gene that forms an autoregulatory loop (1). Surprisingly, however, recently generated knock-in (KI) mice expressing mutant p53 R172H in all tissues clearly establish that mutant p53 is inherently unstable in normal cells. Despite impairment of mutant p53 to transcriptionally induce MDM2, only tumors but not normal tissues of these mice display constitutive stabilization of mutant p53 (2–4). Expression of murine and human MDM2 is controlled by 2 different promoters: the constitutive p53-independent P1 promoter and the p53-responsive P2 promoter (5). Thus, in these KI mice, the constitutive p53-independent transcription of MDM2 from the P1 promoter alone is apparently sufficient to degrade mutant p53 in normal tissues. This finding eliminates the notion that transcriptional inability of mutant p53 to induce sufficient levels of MDM2 is the sole or even primary cause for mutant p53 hyperstability. Rather, on malignant conversion, some undefined additional alteration(s) must occur that stabilize mutant p53.

Compared with p53 null mice, mutant p53 KI mice show an oncogenic gain of function (GOF) phenotype (2, 3). In agreement, depletion of mutant p53 by siRNA in human tumor cells leads to suppressed tumor growth in culture and
in xenografts and to enhanced chemosensitivity (6, 7). Importantly, mutant p53 hyperstabilization is critical for manifestation of its GOF in vivo. In support, constitutive MDM2 deficiency in p53 R172H/R172H mice (in short p53H/H mice) causes earlier tumor onset, increased tumor incidence and metastasis, and shortened survival compared with MDM2-proficient p53H/H mice, implying that GOF depends on mutant p53 levels (4). Thus, tumor-specific stabilization of mutant p53 is a critical determinant of its GOF.

Little conclusive insight currently exists about the precise mechanisms responsible for dysregulating mutant p53 protein levels in cancer cells. In fact, this important question constitutes a major unexplored area in the p53 field, with the exciting prospect that advances have high translational potential that might be exploited for a mutant p53–directed cancer strategy. The existing studies provide only fragmentary insights mostly derived from ectopically overexpressed mutant p53, analyzed in constructed nonphysiologic cell systems of wild-type p53 or null p53 background. In contrast to wild-type p53, many mutant p53 protein species are conformationally aberrant. To prevent aggregation, this dictates their engagement in stable complexes with Hsp90, or with a pharmacologic Hsp90 inhibitor, against Hsp90, or with a pharmacologic Hsp90 inhibitor, destroys the complex, liberates mutant p53, and reactivates endogenous MDM2 and CHIP for mutant p53 degradation.

**Materials and Methods**

**Human cancer cells**

Cancer cell lines MCF7 (breast), RKO and HCT 116 (colon), and U2OS (osteosarcoma), as well as immortalized MCF10A (breast) and MRC5 (diploid fibroblasts), contain functional wild-type p53. Conversely, breast cancer MDA 231 (R280K), MDA 468 (R273H), T47D (L194F), and SK-BR3 (R175H), prostate cancer DU145 (P223L, V274F), pancreatic cancer PANC1, bladder cancer 5637 (R280T), and ovarian cancer EB2 cell lines all harbor mutant p53. Stable mutant SW480 cells (p53 R273H/P309S) inducibly express shp53 under the control of a tetracycline-regulated promoter on adding tetracycline in culture (1.0 mg/mL) or feeding it to nude mice (2 g/L in drinking water; ref. 7). Stable MDA 231-Luc (control) and MDA 231-shp53 cells were a gift from Dr. S. Deb. Cells were cultured in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium. Where indicated, cells were treated with 25 μmol/L ALLN (Calbiochem) for 3 hours. CHX (cycloheximide; Sigma) was added to the medium (final 50 μg/mL). UbAL (BioMol International), a specific inhibitor of deubiquitinases, was included in all buffers. Treatment with 5 μmol/L 17AAG (17-allylamino-17-demethoxygeldanamycin; LC Laboratories) was for 24 hours, 5 μmol/L camptothecin for 3 hours, and nutlin (20 μmol/L; Sigma) for 24 hours. Cell viability was determined by CellTiter-Blue Assay (Promega) in a 96-well format (10,000 cells/well, seeded 24 hours prior). Proliferation was measured by cell counts. Nude mice were injected subcutaneously with MDA 231- or SW480 shp53 cells or vector controls (106 cells per injection site, 6 sites per mouse). Tumors were harvested after 12 or 20 days.

**Plasmids**

CMV-MDM2 plasmid carrying a neomycin resistance gene (11) was transfected with Lipofectamine (Invitrogen). Stably transfected clones were selected in 700 μg/mL G418 (Gibco). The p53 R280K plasmid was subcloned into a retroviral REBNA puro vector. Phoenix A cells were transfected with nut p53R280K REBNA or empty vector. After 48 hours, supernatants containing the retroviral particles were collected and used to infect T47D cells overnight, followed by puromycin (1 μg/mL) selection 48 hours later.

**RNA interference**

For immunoblot, equal total protein of crude cell lysates (2.5–5 μg) was loaded. When loading was normalized for equal amounts of nonubiquitinated p53, a first quantitation immunoblot was run prior to the second definitive immunoblot. For nuclear/cytoplasmic fractionations the Pierce kit was used. Antibodies were FL393 and DO1 for p53, SMP14 for MDM2 (Santa Cruz), HAUSP (Calbiochem), p14Arf (Abcam), p53 Ser15 (Cell Signaling), MDMX (Bethyl Lab), Hsp70, Hsp90, and histone deacetylase (HDAC; all Affinity Bioreagents), PCNA (proliferating cell nuclear antigen), tubulin, actin, and rabbit IgG (immunoglobulin G; all Sigma). For detecting endogenous complexes, crude lysates were immunoprecipitated with 1 μg of antibody for 2 hours. Beads were washed 3 times with SNNTE plus 2 × radioimmunoprecipitation assay buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, pH 7.4) before immunoblotting. Immunofluorescence was done as described (12).

**Results and Discussion**

**Tumor-derived endogenous mutant p53 shows complete lack of ubiquitination, causing its profound degradation defect**

Although stabilization of mutant p53 was noted previously, the ubiquitination status of endogenous mutant
p53 remains controversial. Although early reports noted higher stability of mutant p53 (13), recent studies using ectopic expression suggest that mutant p53 is more ubiquitinated than wild-type p53 in cancer cells (4, 14, 15). Moreover, until now, studies on the regulation of mutant p53 stability were mostly limited to genetic analysis of KI mice (2–4) or ectopic overexpression of mutant p53 in tumor cells (1, 13, 14). Thus, to characterize the degradation of endogenous mutant p53, we probed a panel of randomly chosen human cancer cell lines expressing either wild-type p53 or mutant p53. Mutant p53 tumor cell lines exhibit a dramatic constitutive p53 stabilization ranging from 10- to 20-fold above wild-type p53 cancer lines (Supplementary Fig. S1A). To compare their p53 ubiquitination status side by side, immunoblots from total cell lysates were normalized for comparable amounts of nonubiquitinated p53. Although wild-type p53 ubiquitination was readily detected, mutant p53 ubiquitination remained undetectable in all lines, even after prolonged exposure (Fig. 1A). Moreover, proteasome inhibition by ALLN treatment led to marked accumulation of ubiquitinated p53 only in wild-type cancer cells, whereas mutant p53 remained nonubiquitinated (Figs. 1B and C, right). Putative mutations in the RING domain of MDM2 were excluded in all 6 mutant p53 lines, eliminating mutational inactivation of MDM2 as possible explanation. Ubiquitinated wild-type p53 mainly localizes to the cytoplasm, whereas the nucleus preferentially harbors nonubiquitinated p53 (refs. 12, 16; Fig. 1C, left). Mutant p53 accumulates mainly in the nucleus. To further exclude that the dramatic accumulation of nonubiquitinated mutant p53 in the nucleus might mask a putative ubiquitinated pool in the cytoplasm, we conducted fractionations. Again, ubiquitinated wild-type p53 resides mainly in the cytoplasm and is stabilized by ALLN-mediated proteasome inhibition. In contrast, mutant p53 is nonubiquitinated in both compartments and remains so even after ALLN treatment. Immunoblots normalized for p53 loading. HDAC and Hsp90 as nuclear and cytoplasmic markers. Abbreviations: wt p53, wild-type p53; mut p53, mutant p53.
Neither HAUSP or p14Arf expression nor p53 modification can explain mutant p53 hyperstability

To gain more insight into the mechanism of mutant p53 stabilization in human cancer, we analyzed MDM2, HAUSP, and p14Arf, key molecules in the regulation of p53 stability. MDM2 is a classic target gene of wild-type p53. As expected, because of the lack of transcriptional activity of mutant p53, levels of MDM2 mRNA and protein were downregulated in mutant p53 cancer cell lines compared with wild-type p53 cancer lines but only by about 2- to 3-fold (Fig. 2A and Supplementary Fig. S1B). Importantly and parallel to normal tissues of mutant p53 knock-in mice which express wild-type–like low levels of mutant p53 (4), although downregulated, all mutant human cancer lines express MDM2 protein constitutively. This renders the complete lack of mutant p53 ubiquitination disproportionately severe and leaves it unexplained.

Because hyperstability of mutant p53 might not only be caused by reduced ubiquitination but also by increased deubiquitination, we analyzed HAUSP, the major p53 deubiquitinase. However, levels of HAUSP and its interaction with p53 were similar in wild-type and mutant p53 cells and thus did not contribute to mutant p53 hyperstability (Fig. 2B and Supplementary Fig. S1C).

p14Arf protein levels, normally undetectable, are elevated in cells with a perturbed p53 signaling axis (17, 18), raising the possibility that the MDM2 antagonist Arf could contribute to hyperstability of mutant p53 (4). We therefore examined Arf levels in mutant and wild-type p53 cells. As expected, Arf was undetectable in all wild-type p53 cells (Fig. 2B, bottom). Although Arf was upregulated in mutant EB2, MDA 435, and DU145 cells, it remained undetectable in T47D, MDA 231, and SK-BR3 cells. This lack of correlation between Arf expression and mutant p53 status indicates that Arf upregulation is not a generic mechanism for hyperstabilization of mutant p53, although it might contribute in individual cancers when deregulated.

Alternatively, it was suggested that constitutively activated DNA damage signaling in mutant p53-harboring tumors could account for stabilization of mutant p53 via chronic Ser15 phosphorylation (19). We therefore assessed Ser15 status in unstressed cell panels. However, the majority of mutant p53 lines and all wild-type p53 lines lacked constitutive Ser15 phosphorylation. Although 2 of the 6 mutant lines did show variably increased phosphorylation (Fig. 2C), genotoxic stress (camptothecin) induced Ser15 phosphorylation similarly in both wild-type p53 and mutant p53 cells (Supplementary Fig. S2A). Thus, this modification does not contribute to generic mutant p53 stabilization. Acetylation of the C-terminal lysines of wild-type p53 via competitive inhibition of their ubiquitination was proposed as an important mechanism for wild-type p53 stabilization.
stabilization in response to DNA damage. Moreover, acetylation of wild-type p53 interferes with its interaction with MDM2 (20). To test whether putative hyperacetylation of mutant p53 may cause its ubiquitination defect, we analyzed the acetylation status of normalized amounts of mutant and wild-type p53. However, mutant p53 cells generally express lower amounts of acetylated p53 than wild-type p53 cells (Fig. 2D), excluding hyperacetylation as a contributor to the specific hyperstability of mutant p53.

Selective impairment of MDM2 E3 ligase activity in mutant p53 but not wild-type p53 cancer cells

Consistent with the lack of ubiquitination, the half-life of mutant p53 is dramatically increased compared with wild-type p53 (Fig. 3A). Moreover, other bona fide substrates of MDM2, that is, MDMX and MDM2 itself, are also more stable in mutant compared with wild-type p53 cancer cells (Fig. 3A) and are insensitive to proteasome inhibition in mutant but not in wild-type p53 cancer cells (Fig. 3B). Thus, major physiologic substrates of MDM2 exhibit degradation deficiencies in mutant p53 cells. During stress, DNA damage induces autoubiquitination and self-degradation of MDM2 as part of the stabilization mechanism of wild-type p53 (21). However, although camptothecin destabilized MDM2 in wild-type p53 cells, this was not the case in mutant p53 cancer cells, again supporting their selectively impaired MDM2 activity (Fig. 3C). Of note, mutant p53 is fully competent for binding to MDM2. We did not observe dramatic differences in the physical interaction between endogenous mutant p53 and MDM2, as reported earlier (13, 14). The amounts of coprecipitated p53 simply reflected the respective steady state levels (Supplementary Fig. S2B). In sum, this strongly suggests that the functional impairment of endogenous MDM2 is a major factor responsible for the aberrant stabilization of mutant p53 in cancer cells.

On the other hand, normal H/H mouse embryonic fibroblasts (MEF) harboring the R172H mutation of p53 (H/H MEFs) properly stabilize mutant p53 in response to genotoxic stress and proteasome inhibition, similar to wild-type p53–harboring MEFs (Fig. 3D). Likewise, mutant p53 also properly stabilizes on irradiation in normal spleen and thymus of H/H mice (4). Together, this confirms that in normal cells, MDM2 retains its ability to control mutant p53 stability, whereas this regulation is lost once cells become transformed.

Of note, supraphysiologic levels of ectopic MDM2 readly degraded endogenous mutant p53 in cancer cells (Fig. 3E, lanes 7 and 8). Conversely, proteasome inhibition by ALLN blocked ectopic MDM2-mediated p53 degradation similarly in mutant and wild-type cells (Fig. 3E, compare lanes 1, 2 with 5, 6), confirming earlier reports (13, 14). This also reaffirms that the defect that causes mutant p53 hyperstability lies with blocked endogenous MDM2 activity and not with its substrate.

To further test the idea that mutant p53–harboring cancer cells suffer from a selective inhibition of their MDM2 activity, we forcibly equilibrated MDM2 levels in mutant and wild-type p53 tumor cells. First, MDM2 levels in wild-type p53 breast cancer cells (MCF7) were downregulated by siRNA to match those of mutant p53 breast cancer cells (MDA 231 and MDA 468; Fig. 4A). If MDM2 levels were the sole determinant as was previously assumed, one would now expect wild-type p53 to hyperstabilize to levels matching those of mutant p53. Surprisingly, however, wild-type p53 stabilized by less than 2-fold in MCF7 cells, far below the approximately 20-fold constitutive stabilization of mutant p53 levels in MDA 231 and MDA 468 cells (Fig. 4A). Similar results were obtained for other wild-type p53 cells (RKO and HCT 116; Supplementary Fig. S1D). Conversely, we corrected the lower MDM2 levels in mutant p53–harboring MDA 231 back to those of wild-type p53–harboring MCF7 cells by generating stable MDM2 clones that express about 2-fold higher MDM2 levels (Fig. 4B). MDA 231 cells do not have elevated p14Arf as shown in Figure 2B, thereby excluding a possible negative effect on MDM2 activity. However, in all successfully established MDA 231 clones, mutant p53 levels remained unaffected and ubiquitination nondetectable, even after challenge with ALLN (Fig. 4B). This is despite the fact that ectopic MDM2 undergoes effective complex formation with endogenous mutant p53 (Fig. 4C). Likewise, ectopic MDM2 and endogenous MDMX again display (self)-degradation defects in all mutant p53 MDM2 clones, judged by the poor (for MDM2) or absent (for MDMX) stabilization after ALLN treatment, in contrast to wild-type p53 MCF7 cells (Fig. 4B). Thus, in contrast to markedly supraphysiologic MDM2 expression (Fig. 3E), physiologic levels of overexpressed MDM2 in mutant p53 cancer cells again are functionally inhibited. This suggests that a saturable cellular mechanism leads to MDM2 inactivation in mutant p53 cancer cells.

Tumor-specific stabilization of mutant p53 is caused in part by the HSP90 molecular chaperone machinery

Guarding the proteome against misfolding, aggregation and illicit interactions induced by proteotoxic stress such as reactive oxygen species (ROS), hypoxia, and acidosis, the heat shock family of molecular chaperones guide proper conformational folding of nascent polypeptide “clients” into mature proteins, assist in the productive assembly of multimeric protein complexes, and regulate the cellular levels of their clients by promoting degradation. Normal chaperone function is subverted during oncogenesis to allow initiation and maintenance of malignant transformation and enable cancer cell survival because cancer cells are in a constant state of proteotoxic stress, both from an adverse microenvironment (hypoxia and acidosis) and from within (conformationally aberrant oncoproteins, high levels of ROS, spontaneous DNA damage, and aneuploidy). Thus, their proteins and, in particular, their oncoproteins require massive chaperone support to prevent aggregation and promote survival (22). Hence, in addition to their oncogene addiction, cancer cells also show addiction to HSPs. Among chaperones, Hsp90 is unique because many of its clients are conformationally labile signal transducers with crucial roles in growth control, cell survival, and development. Most
**Figure 3.** Selective impairment of MDM2 E3 ligase activity in mutant p53 cancer cells. A, bona fide physiologic substrates of MDM2 (p53, MDMX, and MDM2 itself) exhibit degradation deficiencies in mutant p53 (MDA 468) compared with wild-type p53 (HCT 116) cells. CHX chase for the indicated times. Actin, loading control; *, a nonspecific band. B, nonresponsiveness of MDM2 and MDMX to proteasome inhibition and DNA damage in mutant p53 cancer cells indicates selective impairment of the MDM2 E3 ligase activity in mutant p53 cancer cells. Left, MDM2 activity is selectively impaired in mutant p53 cancer cells. Although wild-type p53 cells stabilized MDM2 more than 3-fold on ALLN, no MDM2 stabilization occurred in mutant p53 cells. Right, corresponding densitometry quantitation of relative MDM2 levels is shown. Bottom, in contrast to wild-type p53 cells, MDMX levels failed to stabilize in mutant p53 cells in response to ALLN. Cells treated with 25 μmol/L ALLN for 12 hours followed by immunoblotting. C, DNA damage destabilizes MDM2 because of autoubiquitination in wild-type p53 but not in mutant p53 cancer cells. Camptothecin (CAM; 5 μmol/L) for 2 hours. Immunoblot. D, normal mutant p53–harboring p53H/H MEFs (which express somewhat higher constitutive levels in culture than wild-type p53 MEFs), properly stabilize mutant p53 in response to genotoxic stress and proteasome inhibition. Immunoblots of cells treated with 5 μmol/L camptothecin or 25 μmol/L ALLN for 6 hours. E, endogenous mutant p53 is readily degraded by overexpressed levels of ectopic MDM2. Top, immunoblots after transient transfection of MDM2 with and without 25 μmol/L ALLN for 6 hours. Bottom, immunofluorescence of mutant p53 MDA 468 cells transfected with MDM2 (red). The transfected cell does not exhibit p53 staining (green). A to E, immunoblots, actin, and tubulin as loading controls. Abbreviations: wt p53, wild-type p53; mut p53, mutant p53, DAPI, 4′,6-diamidino-2-phenylindole.
importantly, HSP90 plays a key role in the conformational stabilization and maturation of mutant oncoprotein signaling proteins. These encompass steroid hormone receptors, receptor tyrosine kinases (i.e., HER-2), signaling kinases (Bcr-Abl, Akt, and Raf-1), and mutant p53 (8, 22). Hsp90 is the core protein of the multicomponent chaperone machinery HSP90 (that includes Hsp70 and others), a powerful antiapoptotic system that is highly upregulated and activated in cancer. Hsp90 is a dynamic ATPase. ATP binding to the N-terminal domain of Hsp90 and its subsequent hydrolysis drives a conformational cycle that is essential for the HSP90 chaperone activity. Co-chaperone Hsp40 stimulates the associated Hsp70 ATPase activity; chaperone Hsp70 helps fold nascent polypeptides, and adaptor Hop mediates interaction of Hsp90 with Hsp70 (7). Importantly, upregulation of HSPs and, in particular, Hsp90 is an almost ubiquitous feature of human cancers (22). Moreover, structural and affinity differences exist, as revealed by the fact that Hsp90 purified from tumor cells has a 100-fold stronger binding affinity to small molecule ligands of its ATP-binding pocket than does Hsp90 protein purified from normal cells. Of note, tumor Hsp90 is entirely engaged in multichaperone complexes due to an increased load of mutant clients, whereas normal cell Hsp90 is largely uncomplexed and free (22, 23).

Importantly, many mutant p53 proteins are damaged in their conformation-sensitive core domain and form abundant stable complexes with Hsp90 in tumor cells (8, 10). In contrast, wild-type p53 is unable to form stable Hsp90 complexes and does so only transiently and with a few components. For

Figure 4. MDM2 activity is functionally impaired in mutant p53 cancer cells A and B, decreased levels of MDM2 are not the main determinant of mutant p53 stability in cancer cells. A, MDM2 levels in wild-type p53 breast cancer cells (MCF7) were downregulated by siRNA to match those of mutant p53 cells (MDA 231, MDA 468). However, knockdown of MDM2 in MCF7 cells elevates wild-type p53 levels only by less than 2-fold and does not reach the 20-fold stabilization present in mutant p53 cells. B, conversely, the lower MDM2 levels in mutant p53 MDA 231 cells were corrected back to those of wild-type p53 MCF7 cells. Stable MDM2 clones express 2-fold higher MDM2 levels. However, in all established MDA 231 clones, mutant p53 levels remained unaffected and ubiquitination nondetectable, even after challenge with ALLN (3 independent clones of 7 shown). Cells treated with 25 μmol/L ALLN for 12 hours. MDM2 and MDMX display the same (self)-degradation defect as seen in Figure 3, in contrast to wild-type p53 MCF7 cells. Bottom, densitometry quantitation of MDM2 levels of above blot in the absence or presence of ALLN by densitometry of long and short exposures, respectively. Tubulin, loading control. C, ectopic MDM2 undergoes effective complex formation with endogenous mutant p53. MDM2 immunoprecipitation (IP) from MDA 231–MDM2 clone 21 or wild-type p53 MCF7 cells, followed by immunoblots. PCNA, loading control. Abbreviations: wt p53, wild-type p53; mut p53, mutant p53.
example, the A1–5 fibroblasts expressing the temperatureresistant p53 A135V mutant showed that the HSP90 components Hsp90, Hsp70, cochaperone p23, and cyclophilin 40
only coimmunoprecipitate with mutant p53 (at 37°C) but not with wild-type p53 (at 30°C; refs. 8–10).
This stable mutant p53–specific interaction with HSP90 chaperones in cancer cells has been speculated to be linked to aberrant stabilization of mutant p53. In a preliminary
immunoprecipitation study, Peng and colleagues presented circumstantial evidence, although no direct proof, that
MDM2 might be inactivated by being trapped within a trimeric complex of mutant p53–MDM2–Hsp90 and proposed that
Hsp90 binding conceals the Arf-binding site on MDM2, thereby somehow inhibiting its ligase function (24).
Interpretation of this study, however, was made difficult by the fact that MDM2 was found stabilized in tumor cells with
mutant p53 for reasons that are unclear. Although we and others find MDM2 downregulated in mutant p53 tumor
cells, our findings nevertheless fully endorse that mutant p53 hyperstability in cancer cells is strongly dependent on heat
shock support because it inhibits mutant p53 ligases, as described below. HSF1, the master transcriptional regulator
of the inducible heat shock response, controls all stress-inducible chaperones including HSP90 (25). HSF1 is fre-
quently upregulated in human tumors, and the HSF1-mediated stress response plays a causal, broadly supportive
role in mammalian oncogenesis (22, 25). We find that
shRNA-mediated knockdown of HSF1 in mutant p53 cancer
cells, which in turn downregulates Hsp90 and Hsp70 protein,
induces rapid destabilization of mutant p53 (Fig. S3A and
Supplementary Fig. S3C). Together, our findings indicate
that both MDM2 and CHIP are the major endogenous
E3 ligases for mutant p53 (14, 32, 33). Likewise, pharmacologic inter-
ference with both ligases (combined siRNAs
knockdown (Fig. 5H) and almost completely rescued by
nutlin (Fig. 5G) or by siRNA-mediated MDM2 or CHIP
knockdown (Fig. 5H) and almost completely rescued by
synergistic interference with both ligases (combined siRNAs
and nutlin, Fig. 5H, lane 5). In further support, HSF1
knockdown–mediated mutant p53 degradation is again
partially reversed by nutlin (Supplementary Fig. S3B) and
simultaneous knockdown of either MDM2 or CHIP ligases
(Supplementary Fig. S3C). Together, our findings indicate
that both MDM2 and CHIP are the major endogenous
E3 ligases for mutant p53, although CHIP seems to be the
more effective one. Both are presumably active in normal
cells of p53H/H KI mice harboring mutant p53. In cancer
cells, mutant p53 is trapped in stable interactions with
upregulated and activated HSP90 that effectively inhibits
MDM2 and CHIP activity, leading to its aberrant stabiliza-
tion. Which endogenous ligase(s) are responsible? Our evidence
implies that MDM2 and CHIP reactivation in 17AAG-
mediated degradation. First, 17AAG–reactivated MDM2
leads to self-degradation of MDM2 and its physiologic
substrate MDMX (Fig. 5F). Moreover, 17AAG–mediated
mutant p53 destabilization is partially reversed (rescued) by
nutlin (Fig. 5G) or by siRNA-mediated MDM2 or CHIP
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tion. Ectopic expression studies previously implicated CHIP
as an alternative E3 ligase for ubiquitination and degradation
of mutant p53 (14, 32, 33). Likewise, pharmacologic inter-
ference with Hsp90 by 17AAG reactivates both ligases to
degrade mutant p53. This is similar to the functional
redundancy of chaperone-associated E3 ligases that promote
degradation of glucocorticoid and androgen receptors, as
revealed after deletion of CHIP (34).

17AAG reduces cell viability more profoundly in
mutant p53 compared with wild-type p53 cancer cells
To further test the notion that mutant p53 levels are the
major determinant of its oncogenic GOF and to test the
Inhibition of MDM2 and CHIP by HSP90 is largely
responsible for stabilization of mutant p53
In normal cells, HSP90 chaperones regulate the protein
levels of their clients in part by directly recruiting ubiquitin
ligases and presenting them for proteasome-mediated degra-
dation. The chaperone-dependent E3 ligase CHIP binds to
Hsp70 and is a resident part of the HSP90 complex, normally
promoting degradation of clients such as glucocorti-
coid and androgen receptors, c-ErbB2 (30), and phos-
phorylated tau (31). Importantly, degradative function of
CHIP can become defective in tumors.

As shown above, interference with the HSP90 chaperone
function by 17AAG triggers mutant p53 degradation by
freeing mutant p53 from the Hsp90 complex, which appar-
tently enables the reactivation of E3 ligases (Figs. 5B–J).
Which endogenous ligase(s) are responsible? Our evidence
implies that both MDM2 and CHIP reactivation in 17AAG-
mediated degradation. First, 17AAG–reactivated MDM2
leads to self-degradation of MDM2 and its physiologic
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degradation of glucocorticoid and androgen receptors, as
revealed after deletion of CHIP (34).
Stabilization of mutant p53 in cancer cells is caused by the HSP90 chaperone machinery that inhibits the MDM2 and CHIP E3 ligase activity. A, HSF1 knockdown, which leads to downregulation of Hsp90 and Hsp70 proteins, induces destabilization of mutant p53. The eukaryotic genome contains separate genes encoding constitutively expressed and inducible Hsp90. Only inducible Hsp90 transcription is controlled by HSF1 (23). Note that the Hsp90 antibody recognizes both constitutive and inducible Hsp90, explaining the only partial obliteration of Hsp90 after HSF1 knockdown. B, Hsp90 interference by 17AAG causes release of mutant p53 from the Hsp90 complex. Hsp90 (or IgG control) immunoprecipitation (IP; 1 mg protein each) from MDA 231 cells before and after 17AAG treatment (5 μmol/L for 24 hours). HAUSP, loading control. C, 17AAG induces ubiquitination of mutant p53 in a dose-dependent manner. Cells were treated with the indicated concentrations of 17AAG for 24 hours. D, Left, 17AAG induces degradation of mutant p53 (and MDMX) in a dose-dependent manner. 17AAG induces degradation of mutant but not wild-type p53 in cancer cells. Right, cells treated as indicated for 24 hours; 2 μg of mutant p53 and 20 μg of wild-type p53 cell extracts probed. 17AAG induces degradation of mutant p53 but does not decrease Hsp90 levels. Under normal conditions, HSF1 is also a client of HSP90 but held in an inactive HSF1–HSP90 complex. 17AAG inhibits HSP90 chaperone activity and promotes the release of HSF1 for transcriptional activation of HSPs, including Hsp90. This explains the slight increase in Hsp90. HAUSP, loading control. E, downregulation of Hsp90 protein by siRNA destabilizes mutant p53 in MDA 231 and 5637 cancer cells. Scr si, scrambled siRNA. Three days posttransfection, cells were harvested and analyzed by immunoblots. Actin, loading control. F, 17AAG decreases the half-life of mutant p53, MDM2, and MDMX. CHX chase of MDA 231 and MDA 468. Cells treated with 5 μmol/L 17AAG for 16 hours, then 17AAG plus 50 μg/mL CHX for the indicated times. G, nutlin partially prevents 17AAG-induced destabilization of mutant p53, indicating MDM2 reactivation on 17AAG. PANC1 cells harboring mutant p53 were treated with 5 μmol/L 17AAG with or without 10 μmol/L nutlin for 16 hours. Immunoblot. Actin, loading control. H, siRNA-mediated knockdown of MDM2 and CHIP rescue 17AAG-induced destabilization of mutant p53. Abbreviations: wt p53, wild-type p53; mut p53, mutant p53.
Figure 6. 17AAG reduces cell viability more profoundly in mutant p53 compared with wild-type p53 cancer cells. A to D, knockdown of mutant p53 by shRNA inhibits proliferation and invasion of human cancer cells in vitro and in vivo. shp53-mediated stable knockdown of mutant p53 in MDA 231 compared with parentals, assayed for proliferation in vitro (A) and in tumor xenografts in nude mice (C and D). C, average tumors weight (n = 12 each) at day 20. Bars represent mean ± standard error. All tumors show significant reduction of mutant p53 levels (examples shown). B, knockdown of mutant p53 in MDA 231 cells by tetracycline-inducible shp53 inhibits their invasion. Matrigel Boyden chambers and immunoblot (bottom). D, knockdown of mutant p53 in SW480 colon cancer cells by tetracycline-inducible shp53 inhibits their growth in vivo. Tumor xenografts were harvested at day 12. Bottom, p53 immunostaining (DO1) in parental and knockdown xenografts after oral tetracycline fed to nude mice. The shp53 tumor shown corresponds to the single larger tumor in graph above. E, knockdown of mutant p53 by 17AAG (2 µmol/L) and/or tetracycline-inducible shp53 RNAi decreases cell viability of mutant p53 SW480 cells proportional to the extent of mutant p53 destabilization (bottom). CTB assay. Cells treated with tetracycline for 24 hours or left untreated, then concurrently with empty vehicle and/or 2 µmol/L 17AAG for 24 hours. F, 17AAG inhibits cell viability more profoundly in mutant p53 than in wild-type p53 cancer cells. CTB assay. Cells treated with 2 or 5 µmol/L 17AAG for 24 hours or left untreated. G, 17AAG loses killing efficacy when its ability to degrade mutant p53 is overwhelmed by excess ectopic mutant p53. The indicated T47D cells were treated with 0.625 µmol/L 17AAG or mock-treated for 48 hours. Top, cell viability assay (CTB). Bottom, corresponding immunoblot of empty vector or mutant p53 R280K overexpressing T47D cells. HAUSP, loading control.
Figure 6. (Continued) H, proposed model of regulation of mutant p53 stability by the HSP90 multichaperone machinery in cancer cells (see text for explanation). Abbreviations: mut p53, mutant p53; Tet, Tetracycline.

dependence of established tumors on maintaining these high levels, we evaluated the consequences of downregulating mutant p53 by (i) shp53 and (ii) pharmacologic destabilization via 17AAG. In strong support that GOF indeed depends on highly stabilized mutant p53, we and others consistently find that downregulation of mutant p53 by shRNA strongly inhibits the malignant phenotype of human cancer cells in vitro and in vivo (Figs. 6A–D). For example, stable and tetracycline-inducible knockdown of endogenous mutant p53 in breast (MDA 231) and colon (SW480) cancer cells by shp53 RNAi dramatically inhibits cell proliferation (Fig. 6A) and invasion (Fig. 6B) in culture and strongly inhibits tumor growth in nude mouse xenografts in vivo (Figs. 6C and D). In agreement, mutant p53 knockdown in SK-BR3, HT29, SW480, MiaPaCa-2, and MDA 231 cells also caused strong inhibition in proliferation, clonogenicity, and soft agar assays in vitro (6, 7). It also induced strong chemosensitization toward conventional genotoxic drugs (6, 7) and inhibited metastatic spread in mouse xenografts (35). Collectively, these data imply that tumors are addicted to their high levels of mutant p53 and support the rationale that suppression of mutant p53 levels in vivo might achieve clinically significant effects, particularly when combined with anticancer therapies.

Importantly, we find that destabilization of mutant p53 via Hsp90 interference by 17AAG markedly inhibits the viability of SW480 colon cancer cells (Fig. 6E, compare column 1 with column 3). This is again dose dependent because 17AAG at 2 mmol/L cooperates with further reduction of mutant p53 levels by tetracycline-inducible shp53 in reducing cell viability (Fig. 6E, compare column 2 with column 4). Importantly, in a side by side comparison of mutant and wild-type p53–harboring cancer lines, 17AAG reduces cell viability more profoundly in mutant p53 cancer cells. Moreover, 17AAG at the same effective concentration is nontoxic toward normal cells such as MRC5 (Fig. 6F).

Thus, these data suggest that 17AAG might have more potent anticancer effects in mutant p53 tumors compared with wild-type p53 tumors. In support of a causal link between 17AAG-targeting mutant p53 and 17AAG cytotoxicity, 17AAG largely loses its killing efficacy (Fig. 6G, top) when its ability to degrade mutant p53 is overwhelmed by excess amounts of ectopically expressed mutant p53 (“overstuffed”; Fig. 6G, bottom). At the concentration used, the excessively high level of mutant p53 has exhausted the ability of 17AAG to degrade it and concomitantly squelches the ability of 17AAG to affect cell viability. As expected, 17AAG retains some remnant efficacy, suggesting a partial p53-independent component of 17AAG action.

In sum, given that the tumor-specific aberrant accumulation of mutant p53 is the basis for its GOF in malignancy and chemoresistance (6, 7, 35), understanding its underlying mechanism is critical for therapy for mutant p53–harboring cancers. On the basis of our results, we propose the following model as a likely scenario (Fig. 6H). Normal tissues in p53H/H knock-in mice that harbor missense mutant p53 are able to efficiently control their mutant p53 levels, despite the fact that their MDM2 levels are diminished because MDM2 is only supported by constitutive P1 promoter–driven transcription (4). Of note, mutant p53 tumor cells are facing the same MDM2 situation, that is, lower MDM2 levels that are only P1 promoter driven because of impaired p53 transcriptional activity. Therefore, tumor-specific stabilization of mutant p53 proteins—which contributes to driving the tumor phenotype—largely or exclusively depends on a second alteration that these cells undergo on their transformation. This alteration is the addiction of malignant cells to support from the activated heat shock machinery for their survival. In contrast to wild-type p53, the aberrant conformation of many mutant p53 proteins makes them dependent on heat shock support so that they stably engage in complexes with the highly activated HSP90 chaperone to prevent their aggregation. Intimately linked to this conformational stabilization, however, is the fact that this interaction also acts as a large protective “cage” against degradation, thereby enabling the GOF of mutant p53. The E3 ligases MDM2 and CHIP, which in principle are capable of degrading mutant p53, are also trapped in this complex in an inactive state. Because mutant p53 is fully competent to bind to MDM2, HSP90 likely binds to preexisting mutant p53–MDM2 complexes. Alternatively, chaperone-bound mutant p53 could recruit MDM2. Depleting HSP90 components or binding of 17AAG to HSP90 destroys the complex, releases mutant p53, and enables MDM2/CHIP-mediated degradation. However, although unlikely, formally it cannot be completely excluded that despite the same MDM2 situation as in normal tissues, the lower MDM2 levels in tumor cells might also play a minor role in mutant p53 hyperstability. In aggregate, these data provide encouraging evidence for the possibility of mutant p53–directed anticancer therapy that targets an essential cofactor of its stabilization rather than mutant p53 itself. We present a rationale for further pharmacologic improvement in small molecule inhibitors of HSP90 chaperones. Such drugs, generally well tolerated and some already in clinical trials, might...
represent an attractive mutant p53-targeting strategy for those 50% of cancer patients, particularly when combined with other anticancer agents.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

Molecular Cancer Research

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Mol Cancer Res 2011;9:577-588. Published OnlineFirst April 8, 2011.

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