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Pharmacological Targeting of Mutant p53 - HSF1 Feed Forward Circuit

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Abstract of the Dissertation

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Tumor suppressor protein p53 responds to cellular stress by activating transcriptional and transcription-independent programs that induce apoptosis, growth arrest or senescence. Given its critical tumor suppressing role, p53 is often mutated in human cancers. Once altered by missense mutations in the DNA-binding domain, mutant p53 (mutp53) protein becomes highly stabilized in tumors, which is critical for mutp53′s oncogenic gain-of function. Current insight into the mechanism of tumor-specific stabilization of mutp53 is fragmentary and largely derived from ectopically constructed cell systems. We find that in human cancer cells endogenous mutp53, despite its ability to interact with MDM2, suffers from a profound lack of ubiquitination as the root of its degradation defect. In contrast to wtp53, mutp53 proteins are

conformationally aberrant and form a complex with the Heat shock protein 90 (HSP90) chaperone machinery to prevent its degradation. We show that this interaction inhibits MDM2 and CHIP mediated degradation of mutp53. Interference with HSP90 activity by HSP90 siRNA, HSP90 inhibitor 17AAG or histone deacetylase inhibitor SAHA liberates mutp53 from the inhibitory complex and reactivates endogenous MDM2 and CHIP to degrade mutp53. We also show that SAHA and 17AAG exhibit preferential cytotoxicity for mutant compared to wild-type and null p53 human cancer cells. Loss/gain-of-function experiments revealed that although able to exert multiple cellular effects, the cytotoxicity caused by the drugs is mainly due to their ability to degrade mutp53. Thus, our data identifies 17AAG and SAHA as potential candidates for treatment of mutp53 cancers.

Through studying the stabilizing effects of heat shock proteins on mutp53, we discovered a novel gain of function activity of mutp53, whereby mutp53 provides superior tolerance to proteotoxic stress in cancer cells. We found mutp53 induces stabilization and phosphoactivation of Heat Shock Factor 1 (HSF1). HSF1 is a heat shock transcription factor that acts as a master regulator of the heat shock response. Moreover, mutp53 protein, via direct interaction with activated p-Ser326 HSF1, facilitates HSF1 recruitment to its specific DNA-binding elements and stimulates transcription of heat-shock proteins, including HSP90. In turn, induced HSP90 stabilizes its oncogenic clients including EGFR, ErbB2 and mutp53, thereby further reinforcing oncogenic signaling. Thus, mutp53 initiates a feed forward loop that renders cancer cells more resistant to adverse conditions, providing a strong survival advantage.

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List of Abbreviations

p53	tumor protein p53
wtp53	wild type p53
mutp53	mutant p53
HSP90	heat shock protein 90
HSF1	heat shock factor 1
MDM2	murine double minute 2
MDMX	murine double minute X
17AAG	17-allylamino-17-demethoxygeldanamycin
SAHA	suberanilohydroxamic acid
AKT	serine/threonine protein kinase B
PI3K	phosphatidylinositol-3-Kinase
ERBB2/HER2	Receptor tyrosine-protein kinase erbB-2
EGFR	Epidermal growth factor receptor
ER	estrogen Receptor
PR	progesterone receptor

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I. Introduction

A. The discovery of TP53 as an oncogene

Since its discovery in 1979[1-5], decades of research have proved p53 to be one of the most important tumor suppressor genes in humans. The protein p53, also commonly known as Tumor protein p53 (for historical reasons) or more appropriately Tumor suppressor p53, has been shown to possess many diverse tumor suppressor functions that are essential for the well-being of its host organism. However, despite the fact that p53 has been universally accepted as a tumor suppressor for many decades, it was not until after 10 years of its discovery that scientists have agreed that p53 was a tumor suppressor protein and not an oncogene[6,7]. The cause of this misconception was largely due to the unusual conditions of its initial discovery, as the first form of p53 discovered was not wild type, but a mutated form of p53. In 1979, a series of experiments demonstrated the oncogenic functions of mutant p53. In SV40 transformed cells, it was shown that an unknown 53 kDa protein was able to co precipitate with the large T antigen, both in vitro and in vivo. It was also found that this protein was overexpressed in a large number of different SV40 transformed cells, which lead to the belief that SV40 infection, or transformation of mice cells would cause the induction or stabilization of this 53 kDa protein, now known as p53. Early research done on p53 revealed that when mouse 3T3 cells had their growth arrested by serum deprivation, the cells would have very low p53, both at the mRNA and protein levels. Vice versa, when cells were induced to grow by serum stimulation, the levels of both p53 mRNA and protein would increase[8]. Similar results were obtained with T lymphocytes[9] and fibroblasts[10], showing that p53 expression is always correlated with cell growth. At this point it seemed clear that p53 was indeed a positive regulator for cell proliferation. Later

on, it was also shown that transfection of p53 cDNA into mutant ras expressing rat embryo fibroblasts was able to induce transformation of these cells[6,11]. It was then further shown that p53 alone could immortalize normal rat chondrocytes[12,13]. Together, these experiments led scientists to hold firm on the belief that p53 is an oncogene capable of inducing cancer. It wasn't until later that a series of experiments proved that p53 was actually a tumor suppressor protein, and that all the previous work done which demonstrated oncogenic functions of p53 was all done on mutant p53.

B. p53 as a tumor suppressor gene

One such experiment that hinted at a presence of a mutation in the p53 gene was the finding that a certain p53 cDNA clone (dubbed clone F9), isolated from tumor cells, would not cooperate with oncogenic H-Ras[14]. It was then through comparing a number of murine p53 cDNA clones with the p53 sequences from lower species that researchers were able to conclude that the F9 p53 gene was in fact the wild-type p53[15]. It was further shown that wild type p53 was able to suppress transformation when co-transfected together with oncogenic H-ras and myc[7,15]. This proved beyond any reasonable doubt that wild type p53 was, in fact, a tumor suppressor gene.

It has since then been shown that p53 is responsible for a series of downstream effects, eventually leading the cells to growth arrest, apoptosis, and DNA repair[16]. Given the important functions of p53 as a tumor suppressor protein, it should come as no surprise that p53 is inactivated in most if not all human cancers[17]. The most common form of p53 inactivation is mutation, which has been shown to occur in more than 50% of all human cancers[18]. One possible outcome of p53 mutation is simply the abrogation of wild type p53, through a nonsense or frame shift mutation or deletion. Such a mutation in one allele would impair the cell's ability to mount an effective p53 response. If such a mutation occurs in both alleles, then the cell would essentially become p53 null.

Interestingly, mutations which render the cell p53 null are not commonly seen. The vast majority of p53 mutations are actually missense point mutations, which generate a hyperstable full length mutant p53 protein[18]. Quite often, not only is this mutant product conformationally aberrant and lacking any wild type function, it also exerts dominant-

negative effects over any wild type p53. For p53 to function as a transcription factor, four p53 proteins must form a homotetramer. And while mutant p53 often lose all functions found in the wild type p53 protein, it still retains the ability to participate in tetramer formation. Thus, if a cell were to have both mutant and wild type p53, the mutant p53 would bind to the wild type p53, creating mixed tetramers that are incapable of any wild type p53 function. This is known as the dominant negative effect of mutant p53[19]. In addition to its dominant negative effect is the gain of function of mutant p53. The mutated p53 protein would often display functions of its own, functions that are not shown in the original wild type protein, many of which actively contribute to tumor progression[16,18,20]. Thus it is clear that when compared to a p53 null mutation, a missense mutation that would result in full length mutant p53 protein would be far more useful for the incipient tumor cell, and why p53 was mistaken for an oncogene in the earlier experiments.

C. Mutant p53 Gain of Function and Oncogene addiction

The concept of Mutant p53's gain of function was not introduced until a few years after researchers realized p53 as a tumor suppressor protein. The first experiment to describe gain of function showed that by ectopically overexpressing mutant p53 in cells that are normally p53 null, the cells are able to gain new phenotypes, increasing their ability to form colonies in vitro and tumors in mice[21]. This was confirmed by in vivo experiments using mutant p53 knock in mice, where it was shown that when compared to p53 wild type and null mice, mice expressing mutant p53 display a broader tumor spectrum that is also more aggressive and metastatic[22-25]. In vitro and xenograft experiments have also confirmed mutant p53's ability to drive invasion and motility[26-30]. Broadly speaking, there are two types of mutant p53 mutations, one type is known as the contact mutants, where the mutation acquired does not drastically alter the conformation of the protein; and the other type includes conformation mutants, in which the mutation acquired clearly disrupts the 3D structure of the protein. Both types of mutants, however, have lost their ability to bind to wild type p53 specific DNA sequences. Despite this, both types of mutants are shown to be able to upregulate many proproliferative and antiapoptotic proteins[18,31-37], and also repress the transcription of other genes, many of which are proapoptotic[38-43]. Mechanistically, this is achieved by mutant p53's ability to engage in protein-protein interaction with many different transcription factors and alter their transcriptional activity, including p63 and p73[23,25,26,44-46]. Further study was able to reveal other functions of mutant p53, which include increasing genomic instability, gene amplification[47-50], hindering apoptotic signaling[51-54], and its effect on the TGFb[26,55] pathway. In human lung cancer cells, it

was shown that mutant p53 drives invasion through promoting integrin recycling at the cell surface[28]. Mutant p53 has also been shown to cause cells to exhibit elevated resistance to a variety of pro-apoptotic signals, including those induced by genotoxic drugs[51,52,56]. The speculation has been made that there are many other possible ways in which mutant p53 can manifest itself, and that mutant p53 is very likely to have an opposite effect to that which wild type p53 exerts[57].

D. Hyperstabilization of mutant p53 in tumor cells

Mutant p53's gain of function largely depends on the stabilization of mutant p53 in cancer cells, and many studies have shown that reducing mutant p53 levels through siRNA results in increased apoptosis; suggesting that these cells have become oncogenically addicted to mutant p53 for their survival[58-63].

Despite being able to identify mutant p53 as a critical target for cancer treatment, a successful strategy to target mutant p53 continues to elude us. As mentioned above, mutp53 has multiple gain of function effects in different cancers that promote cancerous growth[64]. Recently, we and others have shown that lowering mutant p53 levels by RNAi can cause human breast cancer cells to die and/or revert into less invasive phenotypes[65-67]. These studies strongly suggest that decreasing mutant p53 levels in tumors might be beneficial to patients with p53 mutations.

It should also be noted that many widely used chemotherapeutic drugs used to treat cancers might cause mutant p53 levels in tumors to accumulate even further[68]. There has not been an extensive study on whether or not increased levels of mutant p53 would be detrimental to the patient or not, although it was shown that mutant p53 expression is able to enhance drug resistance in hepatocellular carcinomas[69]. One recent disconcerting study has shown that patients harboring p53 mutations had an overall decrease in survival after cisplatin based treatment, while patients with wild type p53 reacted favorably and showed an increase in survival[70]. No speculation was offered in this study on why patients with mutant p53 would react negatively to chemotherapy. Other studies show that when treated with low level radiation or doxorubicin, mice

homozygous for mutant p53 have a decreased survival rate compared to p53 null mice[71]. Taken together, this suggests that increased mutant p53 levels are indeed disadvantageous to cancer patients treated with chemotherapy or radiation.

Given its important role as a tumor suppressor protein, the amount of wild type p53 is closely regulated. It is important to note that this close regulation however, is lost on tumor cells expressing mutant p53. Under normal conditions, the protein stability of wild type p53 is strictly regulated by its E3 ubiquitin ligase MDM2, and p53 levels in healthy cells are kept extremely low[72-74]. Upon stress, posttranslational modifications on both p53 and Mdm2 lead to the disruption of the p53/Mdm2 complex and hence accumulation of p53[8]. In tumors with mutant p53, regulation of p53 by MDM2 is strongly impaired. In contrast to other tumor suppressors that become downregulated in tumor cells upon deletion or nonsense mutations, mutant p53 expression is not only retained but becomes constitutively stabilized. This phenotype is so characteristic that accumulation of high amounts of p53 – detectable by immunocytochemistry - serves as a surrogate marker for p53 mutations in tumors. And while much is known about the regulation of wild type p53, relatively little is known about mutant p53 stability. It had originally been hypothesized that this hyperstabilization of mutant p53 occurs simply because of loss of p53-mediated transactivation of Mdm2, a p53 target gene, resulting in unchecked accumulation of p53[73]. However, this is not the case, as shown by knock-in mice harboring mutant p53R172H. In these mice (commonly known as the H/H mice), mutant p53 levels are strictly controlled and kept at wild-type like levels in normal tissue, despite the fact that Mdm2 is indeed expressed at very low - but still detectable levels - throughout the mouse[23,25,75]. It is only in tumors that mutant p53 is stabilized. However,

in H/H; Mdm2-/-mice mutant p53 becomes accumulated in normal tissue[75]. This proves that even when MDM2 is present at very low levels (driven by its constitutive p53-independent P1 promoter), what little Mdm2 is expressed is still very much capable of degrading mutant p53 and keeping its level in check. This also raises an important question about why the regulation of p53 is only lost in tumors, but not in normal tissue.

E. Heat shock Proteins and their function in cancer

‘Heat shock’ molecular chaperones are essential guardians of the proteome against proteotoxic stress. Organisms respond to stress that can damage proteins, including elevated temperature, oxidative stress, low ph, by inducing these heat shock proteins which aid in the folding and refolding of misfolded proteins.[76] HSP90 is the most abundant of all heat shock proteins, comprising roughly 2% of the entire cytosolic protein pool. Many cancer cells have an even higher amount of HSP90, contributing to up to 5% of the total amount of protein[77]. The multi-component HSP90 chaperone, while not an oncogene itself, plays a key role in the conformational stabilization and maturation of mutant oncogenic signaling proteins. In many cases, elevation of heat shock proteins are necessary for the accumulation and overexpression of oncoproteins in cancer. In contrast to normal cells, HSP90 becomes highly activated in tumors and its affinity to its client proteins is greatly increased[78,79]. This dependence on heat shock proteins makes it a suitable target for cancer cell treatment, which has led to the development of drugs aimed at inhibiting HSP90, which causes degradation of the cancer proteome, in turn causing death in cancer cells[80,81]. Importantly, it has been shown that mutant p53 is a client of HSP90[82]. When taking into account the fact that HSP90 directly binds with mutant p53, and the crucial role HSP90 plays in the stabilization and accumulation of other mutant oncogenic signaling proteins, it’s possible that HSP90 plays an equally important role in the hyperstabilization of mutant p53.

F. Mechanism of mutant p53 stabilization and its degradation

This brings me to the core point of my research. It has been shown that mutant p53 with its gain-of-function effect is essential for cancer cells since they are oncogenically addicted to it[28,83]. While studies hint at the possibility that a further genotoxic therapy-induced increase of mutant p53 levels would be detrimental to the patient, current chemotherapy treatments heed no attention to the p53 mutational status and treat patients with the same chemotherapy drugs. There is urgent need for drugs and treatments for cancers expressing mutant p53. In this study, we show that in human cancer cells harboring mutp53, mutp53-HSP90 interaction can block its degradation by inhibiting constitutive MDM2 and CHIP E3 ligase activity. Interference with the HSP90 pathway, using either RNAi against the upstream regulator or against Hsp90, or with a pharmacological Hsp90 inhibitor; destroys the complex, liberates mutp53 and reactivates endogenous MDM2 and CHIP for mutp53 degradation. We find that not only does HSP90 inhibitor 17AAG readily cause degradation of mutant p53, but so does histone deacetylase inhibitor (HDACi) SAHA. Histone deacetylase inhibitors (HDACi) make up one of the most promising classes of new anticancer drugs and currently under intense investigation. HDACi were initially thought to simply ‘normalize’ the widespread transcriptional silencing mediated by the aberrant cancer cell epigenome. However, with increasing understanding of the acetylome and its regulation, a growing number of non-histone targets of HDACs and thus novel targets of HDACi are being identified. Non-histone targets of HDACs include transcription factors (e.g., p53, E2F1, STAT1, NF-kB). In wtp53 tumor cells, HDACi induce p53 hyperacetylation that stabilizes and transcriptionally activates p53 for pro-apoptotic targets. Other important non-histone

targets of HDACs are the specific cytoplasmic proteins a-tubulin, HSP90 and Ku70. The a-tubulin and HSP90 are specific targets of HDAC6 only[84]. HDAC6 is an obligate positive regulator of the multi-component HSP90 chaperone machinery that is required for proper folding of many oncoproteins, including mutp53. Acetylation of its core component, HSP90 protein, by HDAC6 knockdown or HDACi, inactivates its chaperone activity and leads to degradation of its clients HER2, ErbB1, ErbB2, Akt, c-Raf, BCR–ABL and FLT3[85-88].

HDACi show pleiotropic anti-cancer effects. These comprise tumor-cell intrinsic effects of inducing (typically p53 independently) apoptosis, cell cycle arrest and differentiation; and extrinsic effects of inhibiting invasion, tumor vasculature and enhancing the host immune response. Encouragingly, HDACi show a big therapeutic window in cytotoxicity, with transformed cells at least 10 times more sensitive than normal cells. For example, HDACi selectively induce apoptosis in acute promyelocytic leukemic cells but not in normal bone marrow or preleukemic cells that already express the PMLRARa fusion oncoprotein[89]. Thus, HDACi exhibit an excellent toxicity profile. Currently there are over 70 mostly early phase clinical trials ongoing for 11 different HDACi compounds, either alone or in combination with other drugs, covering a broad range of liquid and solid tumors. Suberoylanilide hydroxamic acid (SAHA, or Vorinostat) is the first and currently only FDA-approved HDACi drug (since 2006 for cutaneous T-cell lymphoma). SAHA inhibits class I, II and IV HDACs, including the cytoplasmic HDAC6, a member of class IIb, and therefore can have multiple cellular effects. In cancer cells, SAHA activates apoptosis by Bid cleavage, upregulation of BH3-only proteins like Bim and Bmf,

accumulation of ROS, suppression of Bcl2 and ROS scavengers and TNFa family activation[90].

For mutp53 harboring tumors, we hypothesized that destabilizing mutp53 may provide a novel therapeutic strategy of clinical significance. However, pharmacologically effective mutp53-targeting small molecule approaches have not been properly explored. Here we show that 17AAG and SAHA both show preferential cytotoxicity in mutp53 cancer cells by destabilizing mutp53 through inhibition of the HDAC6-HSP90 chaperone axis. In summary, this data provides encouraging evidence for the feasibility of mutp53-targeted anticancer therapy using a well-tolerated small molecule inhibitor that is already in late clinical trials.

G. Mutant p53 enhances the Heat Shock Response by modulating HSF1 activity

While studying the effects of heat shock proteins on the stabilization of mutant p53 we were also able to discover a novel gain of function activity of mutant p53, which is that it enhances the function of heat shock factor 1 (HSF1). HSF1 is the master transcription factor of many inducible heat shock proteins, including HSP90. In contrast to normal cells, where HSF1 is transiently engaged in conditions of proteotoxic stress, tumor cells are under permanent proteotoxic stress due to adverse tumor environments. Importantly, activation of HSF1 is not only a reflection of the transformed phenotype but also appears to be essential for malignant transformation. Beyond its protein-folding role, HSF1 drives broad cancer-specific transcriptional programs that support numerous oncogenic processes of aberrant cell cycle regulation, signal transduction, translation, metabolism and invasion[76,91]. Importantly, recent studies on HSF1 knockout mouse models provide compelling genetic evidence for the existence of a critical oncogenic cooperation between mutp53 and HSF1. In an HSF1-/- background, mutp53 knockin mice show 70% suppression of tumorigenicity, compared with mutp53 knockin/ HSF1+/+ mice[91]. The biochemical basis of this cooperation, however, is not known. Given the importance of both HSF1 and mutp53 in cancer, we sought to define the mechanistic interplay between HSF1 and mutp53 in cancer cells.

We show that mutp53, via augmented Epidermal Growth Factor Receptor (EGFR and/or ErbB2) signaling, hyperactivates the MAPK and PI3K cascades, which lead to stabilization, phosphorylation and transcriptional activation of HSF1. Moreover, by direct protein–protein interaction with activated HSF1, mutp53 facilitates recruitment of HSF1

to its target promoters and stimulates the HSF1-transcription program, including HSP transcription. In turn, HSPs stabilize their oncogenic clients including EGFR, ErbB2 and mutp53, thereby further reinforcing tumorigenesis. Thus, mutp53 initiates a regulatory feed forward loop that renders cancer cells resistant to proteotoxic stress, providing a distinct survival advantage.

II Results

A. Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells

Tumor-derived endogenous mutp53 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. While early reports noted higher stability of mutp53[84], recent studies using ectopic expression suggest that mutp53 is more ubiquitinated than wtp53 in cancer cells[75,85,86]. Moreover, until now, studies on the regulation of mutp53 stability were mostly limited to genetic analysis of KI mice[75,92,93] or ectopic overexpression of mutp53 in tumor cells[23,84,85]. Thus, to characterize the degradation of endogenous mutp53, we probed a panel of randomly chosen human cancer cell lines expressing either wtp53 or mutp53. Mutp53 tumor cell lines typically exhibit a dramatic constitutive p53 stabilization ranging from 10–20 fold above wtp53 cancer lines (Fig. 1A). To compare their p53 ubiquitination status side-by-side, immunoblots from total cell lysates were normalized for comparable amounts of non-ubiquitinated p53. While ubiquitination of the wild type p53 was readily detected, mutp53 ubiquitination remained undetectable in all lines, even after prolonged exposure (Fig. 1B). Moreover, proteasome inhibition by ALLN treatment led to marked accumulation of ubiquitinated p53 only in wild-type cancer cells, while mutp53 remained non-ubiquitinated (Figs.1C). Putative mutations in the RING domain of MDM2 were excluded in all six mutp53 lines, eliminating mutational inactivation of MDM2 as possible explanation. Ubiquitinated wtp53 mainly localizes to the cytoplasm, while the nucleus

preferentially harbors non-ubiquitinated p53[94,95] (Fig. 1D). Mutp53 accumulates mainly in the nucleus. To further exclude that the dramatic accumulation of non-ubiquitinated mutp53 in the nucleus might mask a putative ubiquitinated pool in the cytoplasm, we performed fractionations. Again, ubiquitinated wtp53 was mainly located in the cytoplasm where it was further stabilized by ALLN (Fig. 1D)[94]. In contrast, cytoplasm and nucleus were completely devoid of ubiquitinated mutp53, and remained unresponsive to ALLN (Fig. 1D). Taken together, this indicates that the severely impaired degradation of mutp53 is due to grossly defective ubiquitination by MDM2 and possibly other related E3 ligases, thereby causing its aberrant stabilization.

Selective impairment of MDM2 E3 ligase activity in mutp53 but not wtp53 cancer cells

Consistent with the lack of ubiquitination, the half-life of mutp53 is dramatically increased compared to wtp53 (Fig. 2A). Moreover, other bona fide substrates of MDM2, i.e. MDMX and MDM2 itself, are also more stable in mutant compared to wtp53 cancer cells (Fig. 2A) and are insensitive to proteasome inhibition in mutant but not in wt p53 cancer cells (Fig. 2B). Thus, major physiologic substrates of MDM2 exhibit degradation deficiencies in mutp53 cells. During stress, DNA-damage induces auto-ubiquitination and self-degradation of MDM2 as part of the stabilization mechanism of wtp53[96]. However, while camptothecin destabilized MDM2 in wtp53 cells, this was not the case in mutp53 cancer cells, again supporting their selectively impaired MDM2 activity (Fig. 2C). Of note, mutp53 is fully competent for binding to MDM2. I did not observe dramatic differences in the physical interaction between endogenous mutp53 and MDM2, as

reported earlier[97,98]. The amounts of co-precipitated p53 simply reflected the respective steady state levels (Fig. 2D). In sum, this strongly suggests that functional impairment of endogenous MDM2 is a major factor responsible for the aberrant stabilization of mutp53 in cancer cells.

On the other hand, normal H/H mouse embryo fibroblasts harboring the R172H mutation of p53 (H/H MEFs) properly stabilize mutp53 in response to genotoxic stress and proteasome inhibition, similar to wtp53-harboring MEFs (Fig. 2E). Likewise, mutp53 also properly stabilizes upon irradiation in normal spleen and thymus of H/H mice[75]. Together, this confirms that in normal cells MDM2 retains its ability to control mutp53 stability, while this regulation is lost once cells become transformed.

Of note, supraphysiologic levels of ectopic MDM2 readily degraded endogenous mutp53 in cancer cells (Fig. 2F, lanes 7 and 8). Conversely, proteasome inhibition by ALLN blocked ectopic MDM2-mediated p53 degradation similarly in mutant and wild-type cells (Fig. 2F, compare lanes 1, 2 with 5, 6), confirming earlier reports[97,98]. This also reaffirms that the defect that causes mutp53 hyperstability lies with blocked endogenous MDM2 activity and not with its substrate.

To further test the idea that mutp53-harboring cancer cells suffer from a selective inhibition of their MDM2 activity, I forcibly equilibrated MDM2 levels in mutant and wtp53 tumor cells. First, MDM2 levels in wtp53 breast cancer cells (MCF7) were downregulated by siRNA to match those of mutp53 breast cancer cells (MDA231 and MDA468) (Fig. 3A). If MDM2 levels were the sole determinant, as was previously assumed, one would now expect wtp53 to hyperstabilize to levels matching those of

mutp53. Surprisingly, however, wtp53 stabilized by less than 2-fold in MCF7 cells, far below the ~20 fold constitutive stabilization of mutp53 levels in MDA231 and MDA468 cells (Fig. 3A). Similar results were obtained for other wtp53 cells (RKO and HCT116, Fig. 3B). Conversely, we corrected the lower MDM2 levels in mutp53-harboring MDA231 back to those of wtp53-harboring MCF7 cells by generating stable MDM2 clones that express about 2-fold higher MDM2 levels (Fig. 3C). However, in all successfully established MDA231 clones, mutp53 levels remained unaffected and ubiquitination non-detectable, even after challenge with ALLN (Fig. 3C). This is despite the fact that ectopic MDM2 undergoes effective complex formation with endogenous mutp53 (Fig. 4C). Likewise, ectopic MDM2 and endogenous MDMX again display (self)-degradation defects in all mutp53 MDM2 clones, judged by the poor (for MDM2) or absent (for MDMX) stabilization after ALLN treatment, in contrast to wtp53 MCF7 cells (Fig. 4B). Thus, in contrast to markedly supraphysiologic MDM2 expression (Fig. 2F), physiologic levels of overexpressed MDM2 in mutp53 cancer cells again are functionally inhibited. This suggests that a saturatable cellular mechanism leads to MDM2 inactivation in mutp53 cancer cells.

Tumor-specific stabilization of mutp53 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, 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 since cancer cells are in a constant state of proteotoxic stress, both from an adverse microenvironment (hypoxia, acidosis) as well as from within (conformationally aberrant oncoproteins, high levels of ROS, spontaneous DNA damage, aneuploidy). Thus, their proteins and in particular their oncoproteins require massive chaperone support to prevent aggregation and promote survival[99]. Hence, in addition to their oncogene addiction, cancer cells also show addiction to heat shock proteins. Among chaperones, heat shock protein 90 (Hsp90) is unique because many of its clients are conformationally labile signal transducers with crucial roles in growth control, cell survival and development. Most importantly, HSP90 plays a key role in the conformational stabilization and maturation of mutant oncogenic signaling proteins. These encompass steroid hormone receptors, receptor tyrosine kinases (i.e. HER-2), signaling kinases (Bcr-Abl, Akt, Raf-1), and mutant p53[82,99]. Hsp90 is the core protein of the multi-component chaperone machinery HSP90 (that includes Hsp70 and others), a powerful anti-apoptotic 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[42]. Importantly, upregulation of HSPs and in particular Hsp90 is an almost ubiquitous feature of human cancers[99]. 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 mulichaperone complexes due to an increased load of mutant clients, whereas normal cell Hsp90 is largely uncomplexed and free[99,100].

Importantly, many mutp53 proteins are damaged in their conformation-sensitive core domain and form abundant stable complexes with Hsp90 in tumor cells[82,100]. In contrast, wild-type p53 is unable to form stable Hsp90 complexes and does so only transiently and with a few components. For example, the A1-5 fibroblasts expressing the temperature sensitive p53 A135V mutant showed that the HSP90 components Hsp90, Hsp70, cochaperone p23 and cyclophilin 40 only co-immunoprecipitate with mutant p53 (at 37°C) but not with wild-type p53 (at 30°C)[82,101,102].

This stable mutp53-specific interaction with HSP90 chaperones in cancer cells has been speculated to be linked to mutp53's aberrant stabilization. In a preliminary immunoprecipitation study, Peng et al. presented circumstantial evidence, although no direct proof, that MDM2 might be inactivated by being trapped within a trimeric complex of mutp53-MDM2-Hsp90, and proposed that Hsp90 binding conceals the Arf-binding site on MDM2, thereby somehow inhibiting its ligase function[103]. 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. While we and others find MDM2 downregulated in mutp53 tumor cells, our findings nevertheless fully endorse that mutp53 hyperstability in cancer cells is strongly dependent on heat shock support

because it inhibits mutp53 ligases, as described below. HSF1, the master transcriptional regulator of the inducible heat shock response, controls all stress-inducible chaperones including HSP90[104]. HSF1 is frequently upregulated in human tumors and the HSF1-mediated stress response plays a causal, broadly supportive role in mammalian oncogenesis[99,104]. We find that shRNA-mediated knockdown of HSF1 in mutp53 cancer cells, which in turn downregulates Hsp90 and Hsp70 protein, induces rapid destabilization of mutp53 and reduces its half-life (Fig. 4A). Moreover, this relationship follows a direct dose-response. The stronger the HSF1 knockdown and therefore the Hsp90 and Hsp70 knockdown (obtained by repeat rounds of retroviral HSF1-shRNA infection), the stronger the mutp53 destabilization (Fig. 4A).

ATP binding to the N-terminal domain of Hsp90 with subsequent ATP hydrolysis drives a conformational cycle that is essential for HSP90's client binding and chaperone activity. Specific Hsp90 inhibitors such as the ansamycin antibiotics geldanamycin and 17AAG competitively bind to the N-terminal ATP-binding pocket and stop the chaperone cycle, leading to client protein degradation. 17AAG is a potent and highly specific Hsp90 inhibitor currently in phase I-III clinical trials for refractory multiple myeloma and several solid cancers including breast cancer[80]. We find that in all mutp53 human cancer cells tested, 17AAG specifically induces endogenous mutp53 to be released from HSP90 (e.g.Fig. 4B), followed by its efficient ubiquitination (Fig. 4C) and degradation (Fig. 4D)[100,101]. To further support the specificity of the 17AAG data, we examined the effect of Hsp90 levels on mutp53 stability. Indeed, downregulation of Hsp90 protein by siRNA destabilizes mutp53 in MDA231 and 5637 cancer cells (Fig. 4E). Of note, 17AAG does not downregulate Hsp90 levels in mutp53 cells (Fig. 4D). Accordingly, 17AAG markedly

shortens the half-life of mutp53 (Fig. 4F). Interestingly, this is not the case for cancer cells with wild-type p53. In fact, 17AAG has opposite effects, downregulating mutp53 while upregulating wtp53 (Fig. 4D). This 17AAG effect on wtp53 is consistent with previous data[105]. In primary tumor cells derived from mouse models of medulloblastoma that either retain or are null for wtp53 function, inhibition of Hsp90 by a derivative molecule, 17DMAG, induced wtp53-dependent apoptosis[106].

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 degradation. The chaperone-dependent E3 ligase CHIP (carboxy-terminus of Hsp70-interacting protein) binds to Hsp70 and is a resident part of the HSP90 complex, normally promoting degradation of clients such as glucocorticoid and androgen receptors, c-ErbB2[107] and phosphorylated tau[108]. Importantly, CHIP's degradative function can become defective in tumors.

As shown above, interference with the HSP90 chaperone function by 17AAG triggers mutp53 degradation by freeing mutp53 from complexation with Hsp90, which apparently enables the reactivation of E3 ligases (Figs. 4B-F). Which endogenous ligase(s) are responsible? Our evidence implicates both MDM2 and CHIP reactivation in 17AAG-mediated degradation. First, 17AAG-reactivated MDM2 leads to self-degradation of MDM2 and its physiological substrate MDMX (Fig. 4F). Moreover, 17AAG-mediated mutp53 destabilization is partially reversed (rescued) by Nutlin (Fig. 4G), or by siRNA-

mediated MDM2 or CHIP knockdown (Fig. 4H), and almost completely rescued by synergistic interference with both ligases (combined siRNAs and Nutlin, Fig. 4H, lane 5). In further support, HSF1 knockdown-mediated mutp53 degradation is again partially reversed by Nutlin (Fig. 4I) and simultaneous knockdown of either MDM2 or CHIP ligases (Fig 4J). Together, our findings indicate that both MDM2 and CHIP are the major endogenous E3 ligases for mutp53, although CHIP appears to be the more effective one. Both are presumably active in normal cells of p53H/H KI mice harboring mutp53. In cancer cells, mutp53 is trapped in stable interactions with upregulated and activated HSP90 that effectively inhibits MDM2 and CHIP activity, leading to its aberrant stabilization. Ectopic expression studies previously implicated CHIP as an alternative E3 ligase for ubiquitination and degradation of mutp53[98,109,110]. Likewise, pharmacological interference with Hsp90 by17AAG reactivates both ligases to degrade mutp53. 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[111].

17AAG reduces cell viability more profoundly in mutp53 compared to wtp53 cancer cells

To further test the notion that mutp53 levels are the major determinant of its oncogenic gain-of-function (GOF) and to test the dependence of established tumors on maintaining these high levels, we evaluated the consequences of downregulating mutp53 by i) shp53 and ii) pharmacological destabilization via 17AAG. In strong support that GOF indeed depends on highly stabilized mutp53, we and others consistently find that

downregulation of mutp53 by shRNA strongly inhibits the malignant phenotype of human cancer cells in vitro and in vivo (Figs. 5A-D). For example, stable and tetracycline-inducible knockdown of endogenous mutp53 in breast (MDA 231) and colon (SW480) cancer cells by shp53 RNA interference dramatically inhibits cell proliferation (Fig. 5A) and invasion (Fig. 5B) in culture, and strongly inhibits tumor growth in nude mouse xenografts in vivo (Figs. 5C, D). In agreement, mutp53 knockdown in SKBr3, HT29, SW480, MiaPaCa-2 and MDA231 cells also caused strong inhibition in proliferation, clonogenicity and soft-agar assays in vitro[42,112]. It also induced strong chemosensitization towards conventional genotoxic drugs[42,112] and inhibited metastatic spread in mouse xenografts[26]. Collectively, these data imply that tumors are addicted to their high levels of mutp53 and support the rationale that suppression of mutp53 levels in vivo might achieve clinically significant effects, particularly when combined with other anti-cancer therapies.

Importantly, we find that destabilization of mutp53 via Hsp90 interference by 17AAG markedly inhibits the viability of SW480 colon cancer cells (Fig. 5E, compare column 1 with 3). This is again dose-dependent, since 17AAG at 2 mM cooperates with further reduction of mutp53 levels by tetracycline-inducible shp53 in reducing cell viability (Fig. 5E, compare column 2 with 4). Importantly, in a side-by-side comparison of mutant and wild-type p53 harboring cancer lines, 17AAG reduces cell viability more profoundly in mutp53 cancer cells. Moreover, 17AAG at the same effective concentration is non-toxic towards normal cells such as MRC5 (Fig. 5F). Thus, this data suggests that 17AAG might have more potent anticancer effects in mutp53 tumors compared to wtp53 tumors. In support of a causal link between 17AAG targeting mutp53 and 17AAG

cytotoxicity, 17AAG largely loses its killing efficacy (Fig. 5G top) when its ability to degrade mutp53 is overwhelmed by excess amounts of ectopically expressed mutant p53 (‘overstuffed’) (Fig. 5G bottom). At the concentration used, the excessively high level of mutant p53 has exhausted 17AAG’s ability to degrade it and concomitantly squelches 17AAG’s ability 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 mutp53 is the basis for its GOF in malignancy and chemoresistance[26,42,112], understanding its underlying mechanism is critical for therapy of mutp53-harboring cancers. Based on our results we propose the following model as a likely scenario (Fig. 5H). Normal tissues in p53H/H knockin mice that harbor missense mutant p53 are able to efficiently control their mutp53 levels, despite the fact that their MDM2 levels are diminished since MDM2 is only supported by constitutive P1 promoter-driven transcription[75]. Of note, mutp53 tumor cells are facing the same MDM2 situation, i.e. lower MDM2 levels that are only P1 promoter-driven due to 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 upon 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 mutp53’s GOF. The E3 ligases MDM2 and CHIP, which in principle are capable of degrading mutp53, are also trapped in this complex in an inactive state. Since mutp53 is fully competent to bind to MDM2, HSP90 likely binds to pre-existing mutp53-MDM2 complexes. Alternatively, chaperone-bound mutp53 could recruit MDM2. Depleting HSP90 components or binding of 17AAG to HSP90 destroys the complex, releases mutp53 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.

B. SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis

SAHA downregulates mutp53 but not wtp53

Similar to the effects of 17AAG on mutant p53, the effects of SAHA on mutant p53 occurs at the level of protein degradation but not at the level of transcription. To analyze whether SAHA has an effect on p53 expression, a panel of human tumor cell lines harboring either mutant or wild-type p53 were analyzed by immunoblots. As shown in Figure 6A, SAHA strongly downregulates the various aberrantly accumulated mutp53 proteins in all cases, but does not alter levels of wtp53 protein. Also, SAHA treatment decreases the levels of MDM2 and MDMX, all bona fide physiologic substrates of MDM2 in mutant, but not wtp53 cells (Fig. 6A). Moreover, SAHA-mediated downregulation of mutp53 by low micromolar concentrations is dose-dependent and correlates with induction of PARP cleavage in cancer cells (Fig. 6B). Furthermore, consistent with mutant-specific downregulation, SAHA induces ubiquitination of mutant but not wtp53 (Fig. 6F). Importantly, proteasome inhibition by MG132 completely rescues SAHA-mediated downregulation of mutp53, MDM2 and MDMX, indicating that SAHA regulates their stability on the post-transcriptional level (Fig. 6C). To further explore the mechanism of SAHAinduced destabilization of mutp53, we next performed cycloheximide (CHX) chase experiments. As shown in Figure 6D, SAHA dramatically decreases the half-life not only of mutp53 protein, but also of MDMX and of MDM2 itself. Moreover, proteasome inhibiton completely rescues SAHAinduced destabilization of mutp53, MDM2 and MDMX after CHX treatment (Fig. 6D). Conversely, mutp53 cells

were treated with SAHA plus a-amanitin, a potent transcriptional inhibitor that blocks RNA polymerase II. However, transcriptional inhibition does not interfere with SAHA mediated downregulation of mutp53 in all tumor lines tested (Fig 6E). Taken together, our results indicate that SAHA's effect is specific for mutp53, and is largely posttranslational at the level of protein degradation.

SAHA-induced degradation of mutp53 is mediated by reactivation of MDM2 and CHIP E3 ligases

Next we sought to identify the specific E3 ubiqutin ligase(s) that are responsible for SAHA's effect on mutp53. We focused on MDM2 and CHIP, as both proteins have been established as E3 ligases for mutant p53 and the functional inactivation of these two ligases is the cause for aberrant stabilization of mutp53 in cancer cells[65]. Stable overexpression of MDM2 enhances SAHA induced degradation of mutp53 (and of MDMX) compared with vector controls (Fig. 7A). Moreover, the specific MDM2 inhibitor Nutlin partially prevents SAHA-induced destabilization of mutp53 (Fig. 7B). Furthermore, direct siRNA-mediated knockdown of MDM2 and CHIP partially rescues SAHA-induced destabilization of mutp53 (Figures 7C and 10E). Taken together, these data imply that mechanistically SAHA causes mutp53 degradation by inducing functional reactivation of MDM2 and CHIP.

SAHA destabilizes mutp53 by inactivating HDAC6 which inactivates HSP90

Mutp53 KI mouse models definitively clarified that p53 missense mutations alone do not confer aberrant p53 protein stability, as normal tissues of these mice exhibit low, wild-

type like p53 levels[23,25]. Thus, those models showed that hyperstability is not due to loss of transcriptional activation of the p53 target gene MDM2, the long favored proposed mechanism for hyperstability. Instead mutp53 stabilization reflects a tumor-specific activation of a p53-stabilizing pathway. The identity of this pathway seems to be linked to the HSP90 chaperone machinery, which is highly and ubiquitously upregulated specifically in cancer cells. Mutp53 proteins are damaged in their conformation-sensitive core domain. In fact, in tumor cells mutp53 forms stable complexes with Hsp90. These large stable heterocomplexes were previously proposed to inhibit proteasome-mediated degradation of mutp53[100].

HSP90 chaperone activity is regulated by acetylation/deacetylation of Hsp90. HSP90 acetylation at K294 inactivates its chaperone activity by inhibiting its cyclical binding to client proteins and cochaperones. HDAC6 is the positive regulator of the HSP90 chaperone activity by mediating K294 deacetylation[113,114]. Indeed, HDAC6 pharmacological inhibition or HDAC6 siRNA-mediated knockdown leads to degradation of mutp53. As shown in Figure 8A, HDAC6 inhibition by Sulfaraphane (SFN)[115] destabilizes mutp53 (and Mdmx).

Conversely, Nutlin (Fig. 8B) and MDM2 siRNA (Fig. 8C) partially prevent SFN-induced degradation of mutp53, again implicating MDM2 in SFN-mediated degradation. More importantly, HDAC6 inhibition by siRNA specifically destabilizes mutp53 (but not wtp53, that is in HCT116 cells) (Fig. 8D) and this siHDAC6 effect is again rescued by Nutlin or siRNA against MDM2 and CHIP (Fig. 8E). Conversely, HDAC6 overexpression stabilizes mutp53 (Fig. 8F, middle lane). Importantly, this stabilization is largely rescued

by concomitant treatment with SAHA (Fig. 8F, third lane). Furthermore, SAHA targets all HDAC isoforms including HDAC6.16 Importantly, SAHA releases mutp53 by inhibiting the complex between Hsp90 and its p53 client (Fig. 8G). To further support the fact that SAHA specifically works through Hsp90, we examined the effect of Hsp90 silencing. Indeed, although downregulation of Hsp90 by siRNA destabilizes mutp53 (Fig. 8H, compare lanes 1 and 2), SAHA does not induce further destabilization in HSP90-ablated cells (Fig. 8H, compare lanes 3 with 4). Thus, my aggregate results of Figures 1–3 concerning the mechanism of SAHA-induced degradation of mutp53 strongly suggest the following chain of events: in the context of mutp53, to a large (albeit not exclusive) extent SAHA works by inhibiting HDAC6 deacetylation. This leads to inactivation of Hsp90 and release of mutp53 that in turn leads to reactivation of MDM2 and CHIP E3 ligases and p53 degradation.

SAHA shows preferential cytotoxicity for mutp53 tumor cells both in vitro and in vivo

My results so far suggest that mutp53-harboring tumor cells might be especially sensitive to SAHA's antitumor effects. To test this notion, we employed multiple death assays to examine side-by-side randomly selected panels of human tumor cell lines representing different p53 mutational status. One study had previously reported that mutp53 was associated with increased cytotoxicity to the HDAC inhibitors FR901228 (aka FK-228 or depsipeptide) and trichostatin A (TSA)[116]. However, both drugs are of different chemical classes than SAHA and were reported to somehow 'pharmacologically rescue' mutp53 by inducing a wild-type p53-like transcriptional

response, indicated by p21 and MDM2 target gene induction. Because mutp53 is highly expressed, a sudden restoration of wild-type p53 functions was proposed to be the basis of cytotoxicity for these two HDACi[116]. However, in contrast to FR901228 and TSA, SAHA does not restore wild-type p53-like functions in mutp53. In SAHA-treated MDA231 cells, MDM2 fails to be induced by SAHA. p21, although mildly increased, is induced in a p53-independent manner, as indicated by the highest p21induction in p53 null cells (HCT116-/-), and comparable (to mutp53 cells) p21 induction in wtp53 cells (RKO) (Fig. 9A). SAHA is well known to induce p21 (CDKN1A)[89,117,118]. Furthermore, in contrast to wtp53 cells we did not observe upregulation of Puma, Noxa and Bax in mutp53 cells following SAHA treatment (Fig. 9A).

Concerning anti-tumor effects, SAHA indeed shows preferential cytotoxicity in mutp53 cancer cells. SAHA induced strong cell killing in human tumor cells harboring mutp53, as measured by trypan blue exclusion assays (Fig. 9D), cell viability assays (Fig. 9C) and subG1 fractions in FACS analysis (Fig. 9D). In contrast, wtp53 or p53 null human tumor cells show only minimal cytotoxic responses to SAHA (Fig. 4B–D). This result was further supported by determination of IC50s (inhibitory concentrations at which 50% killing is achieved), which was profoundly lower in mutp53 cancer cells (Fig. 9 Table 1). Moreover, as SAHA is an effective HDAC6 inhibitor, which is a positive regulator of the HSP90 chaperone complex, we predicted that in the context of mutp53, SAHA might synergize with a direct Hsp90 inhibitor. Highly specific Hsp90 inhibitors such as the geldanamycin and its clinically active derivative, the prototype 17AAG, competitively bind to the N-terminal ATP-binding pocket and stop the chaperone cycle, leading to client protein degradation[99,100]. Indeed, 17AAG alone shows similar preferential efficacy

in mutp53 tumor cells as SAHA alone. Moreover, co-treatment of SAHA plus 17AAG causes a synergistic loss of cell viability in some mutp53 cancer cells (MDA231), but much less so or not at all in wtp53 cancer cells. This is very obvious in MDA231 and T47D cells that were killed with 100% efficiency by the drug combination (Fig. 9E, right). This synergistic efficacy correlates with the degree of mutp53 destabilization and PARP cleavage in mutant but not wild-type cells (Fig. 9E, left). Of note, SAHA alone induces mutp53 destabilization at least as efficiently as 17AAG alone. Thus, molecularly the synergism of SAHA + 17AAG is explained by complementary drug targets against distinct components of the HSP90 chaperone machinery, leading to direct and indirect (HDAC6) inhibition of the complex's ability to hyperstabilize mutp53.

SAHA's preferential cytotoxic effect on mutp53-harboring cancer cells is to a significant degree due to its ability to degrade mutp53

My data so far shows that SAHA preferentially kills mutp53 tumor cells and that its cytotoxicity completely correlates with SAHA's ability to degrade hyperstable mutp53 protein, even under conditions of strong transcriptional inhibition. Therefore I asked whether the anti-tumor effect of SAHA is dependent on HSP90-mediated mutp53 degradation and if so, to what degree. To test for dependence, I used complementary gain- and loss-of-function approaches of mutp53 cancer cells and interrogated them in an array of functional assays that included cell death, clonogenicity, invasion and chemosensitization.

As shown in Figure 10, SAHA loses more than 50% of its killing efficacy in mutp53-harboring cancer cells when its target (mutp53) is knocked down by Tet-inducible shp53

RNAi, as shown in SW480 and MDA231 cells (Fig. 10A and B). In both cases, SAHA did retain a small remnant efficacy despite p53 downregulation, which might be due to incomplete p53 elimination, and/or to a p53-independent component of the anti-tumoral SAHA effect. These results were confirmed by long-term clonogenicity assays (Figure 5c). Furthermore, SAHA-induces an invasion block in MDA231 cells that correlates with its degradation of mutp53 (-Tet, Fig. 10B, left and right panels). Conversely, the SAHA-induced invasion block is virtually eliminated when mutp53 is downregulated (+Tet, Fig. 10B, left and right panels). This argues that the invasion block in p53-proficient control MDA231 cells is at least in part due to SAHA-induced HSP90-mediated mutp53 degradation.

In further support of a causal link for SAHA targeting mutp53, SAHA loses killing efficacy when its ability to degrade mutp53 is overwhelmed by excess amounts of ectopically expressed mutp53 (‘overstuffed’). Excess ectopic R280K mutp53 that has exhausted SAHA’s ability to degrade it at the concentration used also squelches SAHA’s ability to induce cell death in T47D and SW480 cells by more than 50% (Fig. 10D). As expected and already seen with the knockdown systems (Fig. 10A-C), SAHA retains some remnant efficacy, suggesting a partial p53-independent component of SAHA action. Most importantly, however, in both experimental conditions cells become partially resistant to SAHA because SAHA is no longer able to downregulate mutp53 levels.

As predicted and already seen above, knockdown of MDM2 by siRNA partially rescues SAHA-induced destabilization of mutp53 (Fig. 7C, 10E) and inhibits the cytotoxic effect of SAHA, indicated by reduced PARP cleavage (Fig. 10E).

SAHA strongly chemosensitizes mutp53 cancer cells and this is due to its ability to degrade mutp53

RNA imediated mutp53 knockdown in human cancer cells was previously shown to cause chemosensitization towards an array of conventional genotoxic drugs[42,45,112], hinting that such cancer cells are addicted to the continuous expression of mutp53. Thus, we tested whether pharmacological degradation of mutp53 via HSP90 targeting by SAHA can also mediate chemosensitization in response to conventional genotoxic drugs such as topoisomerase inhibitor camptothecin. As shown in Figure 5F, this is indeed the case. Although low doses of camptothecin (100 nM) and SAHA (625 nM) alone induces only modest cell killing of 10 and 25%, respectively, in MDA231 cells, SAHA markedly synergizes with camptothecin to cause 100% killing. A similar situation is seen with T47D cells. Of note, this chemosensitization is due to SAHA's ability to degrade mutp53, as excess ectopic mutp53, which overwhelms SAHA, completely squelches this effect (Fig. 10F).

So far, we demonstrate for the first time that the HDAC inhibitor SAHA is able to effectively and specifically downregulate mutp53 by promoting its degradation, while having no effect on wtp53 (Fig. 6). Mechanistically, SAHA and 17AAG acts by disturbing the physical interaction between Hsp90 and mutp53, either directly or indirectly, that is the basis of mutp53 hyperstability. This in turn enables the reactivation of the

endogenous ubiquitin ligases MDM2 and CHIP to mediate mutp53 degradation, thus depriving these tumors of a crucial survival factor (Fig. 7 and Fig. 11). As a result, I find that SAHA shows preferential cytotoxicity for mutp53 tumor cells, whereas wtp53 and p53 null tumor cells are much less sensitive (Fig. 9).

C. Mutant p53–HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress

Mutp53 upregulates the HSF1 protein and augments the heat-shock response

I previously showed that HSF1 controls the stability of mutp53 protein in human cancer cells via activation of Hsp90, which strongly stabilizes mutp53 (Fig. 11)[65,66]. Conversely, here I show that mutp53 also induces HSF1 in all human cancer cell lines and mouse primary cells tested. First, RNAi-mediated depletion of mutp53 in SKBr3 breast cancer cells results in downregulation of the HSF1 protein (Fig. 12A). Next, we generated stable isogenic lines of mutp53 MDA231 breast cancer cells that express either Tet-inducible shp53RNA or excess ectopic mutp53 R280K protein matching its endogenous p53 mutation. Similar to SKBr3 cells, shp53RNA downregulates HSF1 and its transcriptional target Hsp70 in MDA231 cells. Importantly, this effect is further enhanced by HSF1 activation via heat shock (HS), a strong inducer of HSF1 transcriptional activity (Fig. 12B). Conversely, excess ectopic native mutp53 R280K in MDA231 cells further increases the HSF1 targets Hsp70/Hsp27 upon HS (Fig. 12C). Likewise, expression of mutp53R175H in p53 null H1299 cells induces HSF1 and Hsp70/Hsp27 (Fig. 12D), further enhanced by HS at the protein (Fig. 12D) and mRNA (Fig. 15C) levels. Upregulation of HSF1 by mutp53 appears to be generic, as it is observed in response to different p53 mutants (Fig. 12D and E). To confirm these results in mice I generated a novel breast cancer model by introducing the well-characterized p53 R172H allele (H thereafter)[23,25] into MMTV-ErbB2 transgenic mice.13 p53/;ErbB2 littermates served as controls. These mice spontaneously develop mammary tumors. Importantly, compared

with their p53/littermates, primary cultures derived from H/H;ErbB2 mammary epithelial cells (MECs; Figure 5c) and -/+;ErbB2 versus H/+;ErbB2 mammary tumors both show increased levels of the HSF1 protein and its targets (Hsp70, Hsp27; Fig. 11F).

Thus, these data indicate that mutp53 positively regulatesHSF1 levels and activity, and that mutp53-mediated upregulation of HSF1 may constitute a novel gain-of-function activity of mutp53.

Mutp53 promotes HSF1 activation via Ser326 phosphorylation

qRT-PCR analysis demonstrated that in contrast to Hsp70, HSF1 transcripts are not affected by ectopic expression of mutp53 in H1299 cells (Fig. 15C), indicating that mutp53 upregulates HSF1 at the post-transcriptional level. In unstressed cells, HSF1 shuttles between the nucleus and cytoplasm, but localizes predominantly in the cytoplasm, due to sequestration by Hsp90. Upon HS, HSF1 is phosphorylated, liberated from Hsp90, undergoes trimerization and translocates to the nucleus to activate target gene expression by binding to specific heat-shock elements (HSE) in target promoters. Importantly, phosphorylation of HSF1 at Serine 326 (p-Ser326) is pivotal to render HSF1 transcriptionally competent[119]. Furthermore, Ser326 phosphorylation protects HSF1 from polyubiquitination and proteosomal degradation, causing HSF1 to stabilize[91]. Thus, we looked for a correlation between levels of p-Ser326 HSF1 and mutp53. Indeed, HS significantly elevated p-Ser326 HSF1 in total cell lysates of MDA231R280K versus control MDA231 cells (Fig. 13A). Moreover, total HSF1 was also increased in the cytoplasm of unstressed MDA231R280K cells, implying that mutp53 affects basal levels of HSF1 even in the absence of proteotoxic stress (Fig. 13B). HS induced nuclear

translocation and p-Ser326-phosphorylation of HSF1, which was augmented in MDA231R280K compared with control cells (Fig. 13B). Conversely, shRNA-mediated downregulation of mutp53 decreased (mainly cytoplasmic) total HSF1 of unstressed cells and decreased HS-activated nuclear p-Ser326 HSF1 (Fig. 13C). Notably, HSF1 was specifically upregulated by mutant but not wtp53. No increase in HSF1 levels or activation was seen in HCT116 p53/versus p53+/+ cells (Fig. 13D).

Mutp53 interacts with activated HSF1 in the nucleus

The best-described mechanism of mutp53 gain-of-function relates to its ability to interact with other transcription factors and modulate their target gene expression[120]. To test whether this mechanism is also engaged in the mutp53-mediated regulation of the HSF1 transcriptional program, we performed co-immunoprecipitations. Indeed, HS induced a specific mutp53-HSF1 complex in MDA231R280K cells, which contained total and activated p-Ser326 HSF1 (Fig. 14A). Importantly, in reciprocal co-immunoprecipitations from MDA231 cells an endogenous nuclear mutp53-HSF1complex, enhanced by HS, was confirmed (Fig. 14B). To directly establish that nuclear mutp53 mainly interacts with the transcriptionally active form of HSF1, nuclear fractions from MDA231 cells after HS were immunoprecipitated with pan-HSF1- or p-Ser326 HSF-specific antibodies and loading normalized for similar amounts of immunoprecipitated total HSF1. Indeed, mutp53 was predominantly complexed with p-Ser326 HSF1 (Figu. 14C). I failed to detect the wtp53-HSF1 complex in HCT116 p53+/+ cells despite HS, indicating that HSF1 interaction is a mutp53-specific trait (Fig. 14D).

Mutp53 protein enhances binding of HSF1 to HSE elements and stimulates its transcriptional activity

The fact that mutp53 preferably interacts with activated HSF1 (Fig. 14B and C) suggests that it may modulate the transcriptional activity of HSF1. Therefore, I examined the mRNA levels of Hsp70 after shRNA-mediated mutp53 knockdown. As expected, HS-mediated proteotoxic stress strongly induced Hsp70 mRNA (Fig. 15A) and protein (Fig. 12B–D) in MDA231 cells. Importantly, in mutp53-depleted cells mRNA induction was suppressed by 50%, especially upon HS with maximum HSF1 activity (Fig. 15A). These results were confirmed by HSF1 reporter assays using HSE-luciferase (Fig. 15B). Conversely, in H1299 cells expressing mutp53R175H Hsp70, mRNA (Fig. 15C) and protein (Fig. 12D) were induced compared with vector controls, whereas the HSF1 transcript level itself was unresponsive to mutp53 (Fig. 15C). In support, HSE-Luc reporter activation was stimulated by 40% in H1299 cells expressing mutp53R175H compared with controls (Fig. 15D). Likewise, the elevated levels of mutp53 in MDA231R280K cells increased HSE-Luc activity upon HS (Fig. 15E). Thus, mutp53 is an important enhancer of the HSF1 transcriptional activity.

Next, I asked whether mutp53 proteins directly contribute to HSF1's physical recruitment to HSF1-binding sites. Chromatin immunoprecipitation (ChIP) analyses in heatshocked MDA231 and MDA468 cells showed that mutp53 is bound with similar efficiency to Hsp27, Hsp90 and Hsp70 promoters as HSF1 (Fig. 15F). Moreover, HSF1 binding to HSE is mutp53-dependent, as HSF1 recruitment to HSE was greatly enhanced in the MDA231R280K compared with MDA231 control cells (Fig. 15G, lanes

7, 8). On the contrary, HSF1 binding to HSE was reduced in mutp53-depleted cells before (Fig. 15H, lanes 9, 11) and after HS (Fig 15H, lanes 10, 12). Hence, mutp53 stimulates its Ser326 HSF1 phosphoactivation, binds to activated p-Ser326 HSF1 and enhances its transcriptional activity via HSF1 recruitment to its HSE elements. As a result, mutp53 significantly amplifies the heat-shock response.

HSF1 activation by mutp53 renders cells resistant to proteotoxic stress

Next, I asked whether mutp53-mediated HSF1 activation provides a survival advantage to cancer cells by bestowing increased tolerance to proteotoxic stress. First, we tested the thermotolerance of MDA231R280K cells with elevated levels of mutp53 compared with vector-transfected cells. Indeed, HS-stressed MDA231R280K cells exhibited higher levels of activated HSF1 (Fig. 13A and B), with subsequent upregulation of Hsp70/Hsp27 (Fig. 12C). Importantly, MDA231R280K acquired higher thermotolerance, indicated by higher cell viability, compared with MDA231 control cells (Fig. 16A). Whereas downregulation of mutp53 by siRNA decreases levels of activated HSF1 and its transcriptional target Hsp27 and confers increased sensitivity to HS (Fig. 16B). Notable, immortalized mutp53 H/H;ErbB2 mouse MECs also exhibited elevated levels of HSF1 and its targets Hsp70/Hsp27 compared to p53/;ErbB2 MECs. As a consequence, mutp53 MECs developed higher resistance to proteotoxic stress induced by HS or proteasome inhibition than p53 null MECs (Fig. 16C). Importantly, that in addition to upregulation of pro-survival HSPs, HSF1 also coordinates a broad pro-tumorigenic transcriptional network in cancer cells including the inhibition of pro-apoptotic genes[76]. Thus, I tested whether elevated mutp53 levels in MDA231R280K

cells promotes chemoresistance compared to control cells. High levels of mutp53 rendered MDA231R280K cells chemoresistant to the chemotherapeutic Camptothecin, as indicated by increased viability and lack of PARP cleavage (Fig. 16D).

MAPK and PI3K cascades mediate HSF1 activation in a mutp53-dependent manner

To date signaling pathways leading to HSF1 activation by Ser326 phosphorylation are not well understood. To identify how mutp53 enhances Ser326 HSF1 phosphorylation in breast cancer cells, we screened a panel of kinase inhibitors. I identified the selective MEK 1/2 inhibitor U0126[51] and the PI3K inhibitor LY294002[121] as potent suppressors of HSF1 Ser326 phosphorylation (Fig. 17). The levels of total and active HSF1 dramatically decreased in MDA231 cells after U0126 treatment in the absence and presence of HS, causing a parallel decline in Hsp70 and Hsp27 (Fig. 17A). Notably, mutp53 depletion sensitizes cells for UO126's ability to suppress HSF1 activation (Fig. 17B, lanes 4–8, p-Ser326 HSF1), indicating that mutp53 promotes HSF1 activation via MAPK signaling.

Likewise, PI3K inhibitor LY294002 suppressed p-Ser326 and total HSF1 levels (Fig. 17C). Moreover, dual inhibition of PI3K and MAPK cascades by combined drug treatment further impeded HSF1 levels and expression of its target genes upon HS (Fig. 17C, lanes 2, 3). Conversely, the inhibition of the HSF1 response imparted by these drugs was largely rescued by overexpression of mutp53 (Fig. 17C), underlining the importance of mutp53 in stimulating MAPK and PI3K signaling to regulate HSF1 activity. Interestingly, levels of mutp53 were also reduced by UO126 and LY294002 in MDA231

cells (Fig. 17A, C and 18H). Mutp53 levels also decreased upon LY294002/U0126 treatment in primary mutp53 H/H;ErbB2 MECs (Fig. 17D). This effect is likely due to decreased transcriptional activity of HSF1, as our previous studies showed that HSF1 ablation destabilizes mutp53 via Hsp90 inhibition9 (Fig. 12A). Indeed, as a result of HSF1 inhibition, LY294002/U0126 treatment significantly reduced the levels of Hsp90a in mutp53 MECs compared with its minor reduction in p53 null MECs, further supporting the notion that regulation of HSF1 is wired through mutp53 when it is present (Fig. 17D).

Hence, my data indicate that mutp53-mediated PI3K/MAPK hyperactivation is an important signaling axis for HSF1 activation via Ser326 phosphorylation. By stimulating PI3K/MAPK cascades, mutp53 initiates a feed forward loop resulting in activation of HSF1 and upregulation of HSPs, which increase proteotoxic tolerance and further stabilize mutp53.

EGFR/ErbB2 signaling mediates HSF1 activation in a mutp53-dependent manner

The transcriptional program involved in mammary tumorigenesis is often modulated by the Epidermal Growth Factor Receptor family including EGFR and ErbB2. As both PI3K and MAPK cascades induce HSF1 activation (Fig. 17), I hypothesized that HSF1 Ser326-phosphorylation could be regulated by upstream EGFR and/or ErbB2 signaling in a mutp53-dependent manner. To test this notion, I treated SKBr3 cells with dual EGFR/ErbB2 tyrosine kinase inhibitor CP724714[122]. As expected, CP724714 inhibited phosphorylation of ERK/pERK and AKT/pAKT (Fig. 18A). Similar to the effect of the PI3K/MAPK blockade (Fig. 17), EGFR/ErbB2 inhibition by CP724714 also reduced levels of activated p-Ser326 HSF1, leading to a decline in mutp53 levels (Fig. 18A). To

further test our hypothesis, we stimulated EGFR signaling by adding EGF into the medium of serum-starved MDA231 cells. Indeed, EGFR activation by Tyr845 phosphorylation19 not only induces AKT and ERK phosphorylation but also enhances Ser326 HSF1 phosphorylation (Fig. 18B), confirming that HSF1 activation is mediated via EGFR signaling.

As EGFR- and/or ErbB2-mediated downstream signaling are involved in HSF1 phosphorylation, we reasoned that mutp53 might enhance HSF1 activation via stimulating the EGFR/ErbB2 pathways. Thus, I tested the effect of differential mutp53 expression on EGFR and ErbB2 signaling in breast cancer cells. Indeed, mutp53 overexpression in MDA231R280K cells potentiated EGFR signaling, as indicated by enhanced EGFR-Tyr845 phosphorylation after EGF stimulation, compared with control MDA231 cells (Fig. 18C). This effect was concomitant with induction of p-Ser326 HSF1 even in the absence of proteotoxic stress (Fig. 18C), further confirming that HSF1 activation is mediated by EGFR signaling in a mutp53-dependent manner. Likewise, stable overexpression of native p53R175H in SKBr3 increased the level of ErbB2 and pAKT (Fig. 18D). Consistently, siRNAmediated depletion of mutp53 in SKBr3 cells reduced both ErbB2 (Fig. 12A) and EGFR levels and was accompanied by decreased p-Ser326 HSF1 (Fig. 18E). Similar to p53 depletion, HSF1 knockdown also reduced the level of ErbB2 and EGFR (Fig. 12A and 18E), consistent with ErbB2 and EGFR being well-established HSPs clients[123]. In agreement with the effects seen in human cancer cells, the presence of the mutp53 allele in MECs from H/+; ErbB2 mice correlated with increased levels of ErbB2 and higher HSF1 activity, indicated by elevated Hsp70/Hsp27 (Figure 18F, left). Moreover, H/H MECs, even in the absence of the ErbB2 transgene,

showed detectable amounts of ErbB2 and higher HSP levels compared with p53/control mice (Fig. 18F, right). In addition, H/H;ErbB2 established mammary tumor cell lines, showed elevated levels of ErbB2, EGFR and activated HSF1 (indicated by increased Hsp70) compared with p53/;ErbB2 littermate (Fig. 18G). Importantly, dual inhibition of PI3K and MAPK signaling not only affected HSF1 activation (Fig. 17C and 18A) but also decreased EGFR levels in MDA231 cells (Fig. 18G). The fact that this dual inhibition was significantly less pronounced in mutp53-overexpressing MDA231R280K cells (Fig. 18H) implies that mutp53 positively regulates the tyrosine kinase receptors ErbB2 and EGFR,22 and via their downstream signaling affects HSF1 activation.

Together, my studies imply that mutp53 cancer cells enhance HSF1 activation in a feed forward mechanism by deregulating EGFR and ErbB2 receptors. To support this notion in human tumors, we examined tissue microarray of 150 breast cancer biopsies with known molecular status (ER+, PR+, Her2+ or triple negative) and correlated the intensity of p53 staining with the localization/level of activated HSF1. Consistent with my model, we found a clear correlation between p53 and nuclear p-Ser326 HSF1 staining only in strongly (4+) Her2-positive tumors (rho=0.213, P=0.008). No correlation between p53 and p-Ser326 HSF1 staining was found in Her2-negative, ER+, PR+ tumors (rho= 0.243, P=0.932; Fig. 18I).

Thus, the mutp53-HSF1 circuit constitutes a novel mutp53 gain-of-function, whereby mutp53 initiates a feed forward loop that enhances EGFR/ErbB2 signaling and amplifies HSF1-induced transcriptional program, imparting an enhanced proteotoxic defense (Fig. 18J).

III Discussion

Mechanism of mutant p53 stabilization and its pharmaceutical treatment

There is growing evidence that the highly accumulated mutp53 protein, a hallmark of almost 50% of human tumors, is a clinically relevant target for intervention. It is the hyperstability of mutp53 that is the basis for its GOF and dominant-negativity (over wtp53 in case of heterozygosity) that promotes malignancy and chemoresistance[108]. Aberrant accumulation of mutp53 does not occur in normal cells[23,73] but is tumor-specific due to massive upregulation of the multi-component HSP90 chaperone machinery that almost ubiquitously accompanies malignant transformation[42,104,109,110]. RNAi-mediated knockdown of mutp53 was shown to decrease tumor cell proliferation in vitro and in xenografts, inhibit invasion and metastasis, and sensitize tumor cells towards genotoxic therapy[82,102,112,124]. However, exploration of pharmacologically effective small molecule therapy that targets mutp53 degradation has barely begun. Our results here provide significant support for the hypothesis that destabilization of mutp53 is indeed an effective strategy for treating this large group of human cancers and that this can be achieved by pharmacological means that are clinically already well developed. Thus, these data carry tangible translational importance.

Based on my results I propose the following model as a likely scenario (Fig. 5H). Normal tissues in p53H/H knockin mice which harbor missense mutant p53 are able to efficiently control their mutp53 levels; despite the fact that their MDM2 levels are diminished since MDM2 is only supported by constitutive P1 promoter-driven transcription[75]. Of note, mutp53 tumor cells are facing the same MDM2 situation, (i.e. lower MDM2 levels that are only P1 promoter-driven due to 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 upon 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 mutp53's GOF. The E3 ligases MDM2 and CHIP, which in principle are capable of degrading mutp53, are also trapped in this complex in an inactive state. Since mutp53 is fully competent to bind to MDM2, HSP90 likely binds to pre-existing mutp53-MDM2 complexes. Alternatively, chaperone-bound mutp53 could recruit MDM2. Depleting HSP90 components or binding of 17AAG to HSP90 destroys the complex, releases mutp53 and enables MDM2/CHIP-mediated degradation. 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.

We show here that HDAC6, a cytoplasmic non-histone HDAC which deacetylates Hsp90 and functions as an obligate positive regulator of the HSP90 chaperone machinery[113,114], is also critically important in enabling aberrant stability of mutp53. Inhibition of HDAC6, known to maintain Hsp90 chaperone activity towards other cancer-related client proteins such as androgen receptor, estrogen receptor and ErbB2[125],

destabilizes mutp53 by inactivating Hsp90 (Fig. 8). Furthermore, SAHA treatment leads to strong acetylation of HSP90, interfering with its chaperone activity[126]. Importantly, I was able to demonstrate for the first time that the HDAC inhibitor SAHA is able to effectively and specifically downregulate mutp53 by promoting its degradation, while having no effect on wtp53 (Fig. 6). Mechanistically, SAHA acts by inhibiting HDAC6, thereby disturbing the physical interaction between Hsp90 and mutp53 that is the basis of mutp53 hyperstability. This in turn enables the reactivation of the endogenous ubiquitin ligases MDM2 and CHIP to mediate mutp53 degradation, thus depriving these tumors of a crucial survival factor (Fig. 7, see model in Fig. 11). As a result, I find that SAHA shows preferential cytotoxicity for mutp53 tumor cells, whereas wtp53 and p53 null tumor cells are significantly less sensitive (Fig. 9).

This finding is important on several accounts. First, it was previously thought that SAHA-induced tumor cell death was independent of their p53 status. This conclusion, however, needs to be revised as it was largely based on equal efficacy between p53-null and wtp53 tumor cells[90,127,128]. My study, which is the first systematic side-by-side comparison that includes a random selection of established human mutp53 tumor cells, clearly shows a significantly increased responsiveness in mutp53 tumors. Moreover, SAHA is a pleiotropic drug that traditionally is thought to act primarily at the epigenetic transcriptional level[84,90]. However, my gain-and loss-of function studies demonstrate that in the case of mutp53 tumor cells, SAHA's effect is mainly posttranslational. Its cytotoxicity is to a substantial (albeit not exclusive) degree directly and causally linked to its ability to destabilize mutp53 at the protein level.

SAHA induces mutp53 destabilization at least as efficiently as 17AAG, the prototype of direct Hsp90 inhibitors, which until now were the only other class of mutp53 destabilizing agents[65]. Moreover, a synergistic effect of both drugs is seen in some mutant lines that correlates with further decrease of mutp53 levels (Fig. 9E). We propose that inhibiting HDAC6 by SAHA, which in turn causes hyperacetylation of HSP90, further lowers the threshold of inhibiting HSP90 by 17AAG, resulting in enhanced ubiquitination of HSP90 client proteins including mutp53. Cancer cells that overexpress mutp53 are generally highly resistant to conventional chemotherapeutic drugs. Of note, using gain/loss-of-function manipulation, we demonstrate that SAHA, by virtue of depleting mutp53, is able to dramatically chemosensitize mutp53 cells to genotoxic stress agents such as camptothecin (Fig. 10).

Comprehensively, these data provide encouraging evidence for the possibility of mutp53-directed anticancer therapy that targets an essential co-factor of its stabilization rather than mutp53 itself. I present a rationale for further pharmacological improvement in small molecule inhibitors of HSP90 chaperones and HDACs. 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 anti-cancer agents.

Mutant p53 –HSF1 feed forward loop

Inherent to malignant transformation is massive perennial proteotoxic stress due to aneuploidy, ROS, hypoxia and acidosis[75,116]. To overcome proteotoxic stress, cancer cells mount a wide range of adaptive mechanisms in which the HSF1-orchestrated

response plays the central role. Aside from maintaining cellular homeostasis by stress-mediated induction of HSPs[129], the master transcription factor HSF1 coordinates a wide range of fundamental cellular processes that are critical for malignancy including cell cycle control, metastases and inhibition of apoptosis[65]. Not surprisingly HSF1 is upregulated in 80% of breast cancers and is associated with high histologic grade and increased mortality[117]. Although interception of pathways leading to activation of the HSF1- mediated adaptive mechanisms will likely have high therapeutic potential, the molecular mechanisms causing HSF1 activation remain poorly defined.

p53 mutations are the most frequent genetic alterations in breast cancer, such as in ErbB2+ (72%) and triple negative (80%) tumors, and correlate with high rates of metastatic recurrence, chemoresistance and poor overall survival[118]. A critical cancer specific phenotype of mutp53 is increased protein stability causing mutp53 accumulation in tumors, but not in normal tissues[84]. My studies and others showed that cancer-specific accumulation of mutp53 is critical for many aspects of tumorigenesis, and is the key determinant of mutp53's gain-of-function[28,99,101]. Due to its high translational impact, the question of what causes tumor-specific mutp53 stabilization has recently attracted much attention[23]. I have shown that HSF1, by transactivation of the inducible Hsp90, stabilizes mutp53 via Hsp90 complex formation that protects mutp53 from E3 ubiquitin ligase degradation by Mdm2 and Chip[28]. Thus, upregulation of HSF1 in cancer cells can mechanistically underlie the cancer-cell-specific stabilization of mutp53.

Conversely, here I was able to show that mutp53 is also an important determinant of HSF1 function, constituting a positive feedback loop. Mutp53 promotes phosphoactivation and stabilization of HSF1 by stimulation of the EGFR/ErbB2/MAPK/PI3K signaling cascades. Moreover, mutp53 directly interacts with p-Ser326 HSF1 to recruit HSF1 to its specific DNA-binding sites in target gene promoters and enhances its transcriptional program (Fig. 15). As a consequence, mutp53 endows cancer cells with superior resistance to proteotoxic stress and broadly promotes oncogenic signaling via HSF1. These observations may have significant clinical impact, as conventional chemotherapeutics further stabilize already elevated mutp53 levels (Fig. 16D), which inadvertently may stimulate adaptation to adverse environments and promote cancer cell survival and chemoresistance. Thus, the notorious chemoresistance of mutp53 cancer cells[51] can at least in part be attributed to the positive mutp53-HSF1 circuit (Fig. 16D). In contrast, mutp53-destabilizing therapies may impede or at least offset the adaptive responses, or even sensitize tumors to conventional cytotoxic therapies. Interestingly, contrary to mutp53, wtp53 has been shown to negatively regulate cytoprotective function of HSF1 and heat-shock response, which at least in part is mediated by SIRT1, NAD+ dependent deacetylase[130].

How exactly does mutp53 stimulate HSF1 activation? Recently it has been demonstrated that HSF1 activation by Ser326 phosporylation depends on deregulated MAPK signaling[91]. On the other hand, using ectopically expressed mutp53 in H1299 cells, it has been established that mutp53 drives enhanced EGFR recycling to the cell surface of cancer cells[28]. This trafficking activity of mutp53 protein depends on Rab-coupling protein (RCP) and results in constitutive mutp53-driven EGFR signaling.

Sustained EGFR signaling launches PI3K and MAPK intracellular signaling cascades[131]. Indeed, I identified mutp53-driven deregulated MAPK/ERK and PI3K signaling to be important effectors of HSF1 activation (Fig. 17C and D), whereas inhibition of the upstream EGFR and ErbB2 receptors prevented HSF1 activation (Fig. 18A). Importantly, the modulation of mutp53 levels not only affects EGFR activation upon EGF stimulation but also the total amounts of EGFR, suggesting that in addition to the RCP-mediated receptor recycling mechanism, mutp53 may also regulate protein stability of the EGFR (Fig. 18C, G and E) and ErbB2 (Fig. 12A and 18D). Conversely, HSF1 knockdown destabilizes both EGFR and ErbB2 (Fig. 12A and 18D), consistent with the facts that EGFR is an established Hsp90 client[132] and that ErbB2 is stabilized by Hsp90, Hsp70/Hsc70 and Hsp27[129,133]. In total, my results strongly imply that the EGFR and ErbB2 signaling, at least in part, is mediated by mutp53 in an HSF1-dependent manner. In support, I found a correlation between mutp53 and nuclear p-HSF1 levels only in strongly (3+) HER2-positive, but not in HER2-negative, ER/PRpositive human breast cancers (Fig. 18I). Together, my data indicate a strong oncogenic cooperation between mutp53 and EGFR/ErbB2 signaling, and implicating the latter as an important determinant of mutp53 gain-of-function activity.

Thus, I delineate a novel gain-of-function of mutp53 defined by a mechanistic feed forward link between HSF1 and mutp53. I propose that mutp53, through enhanced recycling[28] and/or stability of EGFR and ErbB2, augments MAPK and PI3K signaling, causing phosphoactivation of HSF1. Concurrently, mutp53 via direct interaction with activated HSF1 facilitates binding of HSF1 to its DNA-binding sites and stimulates transcription of HSPs that further stabilize EGFR, ErbB2, mutp53 and other oncogenes,

reinforcing tumorigenesis (Fig. 18J). Hence, mutp53 initiates a feed forward loop that endows cancer cells more resistant to proteotoxic stress, providing a distinct survival advantage.

IV Future Directions

This study provides strong rationale for further testing mutant p53 destabilizing cancer therapy *in vivo*. If it could be confirmed, it would establish mutant p53 as an important *direct* drug target in many cancer patients. To rigorously validate mutant p53 as a target in a native organismal context, a conditionally inactivatable mutant p53 knockin mouse must be generated. While I have already shown that acute removal of mutant p53 in cell cultures and xenograft models result in a loss of cell viability, a conditional inactivatable mutant p53 knockin mouse would be able to confirm my hypothesis that eliminating stabilized mutant p53 has a positive therapeutic effect in vivo, even in the absence of a wildtype allele, since tumor cells show addiction to its gain-of-function.

Additionally, other compounds and drugs that can efficiently cause the downregulation of mutant p53 await to be discovered, especially newer more efficient HSP90 inhibitors and HDAC inhibitors. Early preliminary data that I have obtained with a new generation HSP90 inhibitor Ganetespib showed very promising results, where I was able to achieve the same mutant p53 downregulation and loss of viability *in vitro* as I would with 17AAG or SAHA, but with roughly 100 times less concentrated drug. Drug treatment of mutant p53 knockin mice is also another important step in confirming that eliminating stabilized mutant p53 has a positive effect in patients.

V. Materials and Methods

Human cancer cells

Breast cancer MDA231 (p53R280K), MDA468 (p53R273K), T47D (p53L194F) and SKBR3 (p53R175H), colon cancer SW480 (p53 R273H and P309S), prostate cancer DU145 (p53P223L and V274F), pancreatic cancer PANC1 and ovarian cancer EB2 cell lines harbor mutp53. Conversely, MCF7 (breast), RKO, HCT 116 and DLD1 (colon), U2OS and SJSA (osteosarcoma) contain functional wtp53. SAOS2 and HCT116+/+ cells are p53 null. Stable mutant Tet-On SW480 (p53 R273H/P309S) inducibly express shp53 under the control of a tetracycline-regulated promoter when tetracyclin is added into the culture medium (1.0 mg/ml).28 T47D and SW480 cells stably overexpressing excess ectopic p53R280K were generated by transfection and selection. All cells were cultured in 10% FCS/DMEM. Where indicated, cells were treated with CHX (50 mg/ml, Sigma, St. Louis, MO, USA), MG132 (5 mM) or a-amanitin (10 mg/ml, Sigma) added to the medium. Treatment with SAHA was for 24 h at the indicated concentrations. SFN (20 mM, Sigma) was used for 24 h, 17AAG (5 mM, 17-allylamino-17-demethoxygeldanamycin, LC Laboratories, Woburn, MA, USA) was used for 24 h, 5 mM Camptothecin for 3 h and Nutlin (20 mM, Sigma) for 24 h. Cell death was determined by trypan blue exclusion, subG1 fractions in propidium iodide-stained flow cytometry, CellTiter-Blue cell viability assays (Promega, Fitchburg, WI, USA; 96-well format with 10 000 cells/well seeded 24 h before) and standard clonogenicity assays. Invasion was determined by standard Matrigel Boyden chambers. All cell viability assays were done using CellTiter-Blue Cell Viability Assay (Promega, 96-well format

with 5000 cells/well seeded 24 h before). Cells were treated for 48 h in various concentrations of drug used. Florescence was detected by SPECTRAmax M2 (Molecular Devices, Sunnyvale, CA, USA). IC50 was calculated using GraphPad Prism 5, 2 sites-Fit logIC50 program (GraphPad Prism, La Jolla, CA, USA).

Plasmids

pCMV-MDM2 and pcDNA3.3-mutp53 plasmids carrying a Neomycin resistance gene were transfected with Lipofectamine (Invitrogen, Carlsbad, CA, USA). Stably transfected clones were selected in 700 mg/ml G418 (Gibco, Carlsbad, CA, USA).

RNA interference

Pools of four different siRNA duplexes specific for human HDAC6 (Dharmacon, Lafayette, CO, USA), MDM2 (Ambion, Austin, TX, USA) and CHIP (Dharmacon) or scrambled control duplexes were transfected with Lipofectamine 2000. Cells were harvested 48 h later for analysis. For Hsp90 silencing, MDA231 cells were transfected with 10 pmol of Silencer Select siRNAs (Ambion).

Immunoblots and immunoprecipitations

For immunoblots, equal total protein of crude cell lysates (2.5–5 mg) were loaded. Antibodies were FL393 and DO1 for p53, SMP14 for MDM2 (Santa Cruz, Santa Cruz, CA, USA), MDMX (Bethyl Lab, Montgomery, TX, USA), CHIP (Calbiochem, Darmstadt, Germany), HDAC6 (Santa Cruz) HAUSP (Calbiochem), cleaved PARP (Cell Signaling, Beverly, MA, USA), Hsp90, E-cadherin, pan-p63 H137 (all Santa Cruz), actin and rabbit

IgG (Sigma). For detecting endogenous complexes, crude lysates were immunoprecipitated with 1 mg antibody for 2 h. Beads were washed three times with SNNTE plus 2X RIPA (50mM Tris, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, pH 7.4) before immunoblotting.

ChIP assays

ChIP assays were performed as described in Denissov et al[134]. Antibodies were the following: p53: (DO-1, BD Pharmingen, San Jose, CA , USA), HSF1 (sc9144X, Santa Cruz Biotechnology, Dallas, TX, USA) and IgG (ab46540, Abcam, Cambridge, MA, USA). After purification of the DNA with the Mini Elute Kit (Qiagen), the relative binding of HSF1 and p53 to HSP promoter sites was analyzed using gene-specific primers: Hsp90F: 5'-TTTAAGGCGGAGGGATCTAC-3' , Hsp90R: 5'-TACCCAGACAGTCCCAACAC-3' , Hsp27F: 50-AGTTTCTGAGAGCCCAGACC-30 , Hsp27R: 5'-GCAGGCTGGTAGGGATTAAC-3' and Hsp70F: 5'-CTGTCAATTAGGCGCTGAAG-3', Hsp70R: 5'-TCTTCTGGGATTCACTGGAG-3' and Real-time qPCR analysis. Analysis of the myoglobulin promoter (myoF: 5'-CTCATGATGCCCCTTCTTCT-3'; myoR: 5'-GAAGGCGTCTGAGGACTTAAA-3' served as an internal negative control. The primers to detect Hsp70-specific HSE were F:5'-GAAGACTCTGGAGAGTTCTG-3' and R:5'-CCCTGGGCTTTTATAAGTCG-3'[135].

Cell fractionation

Cells were harvested, rinsed, pelleted, resuspended in 5 vol of cold CARSB buffer (10mM Tris pH 7.5, 1.5mM CaCl2, 10mM NaCl, protease inhibitor cocktail, 10mM

sodium orthovanadate) and allowed to swell on ice for 15 min, after which Triton X-100 was added to a final concentration of 0.3%. The homogenate was spun for 10 min at 1000X g. The supernatant (cytoplasmic fraction) was adjusted to 200mM NaCl. The crude nuclear pellet was suspended in buffer C (10mM Tris pH 7.9, 1.5mM MgCl2, 10mM KCl, 400mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail, 10mM sodium orthovanadate) and sonicated. The homogenate was centrifuged for 15 min at 16000X g. This final supernatant comprises the nuclear fraction.

MEC cultures

Mammary glands were dissected from 8-week-old virgin female mice and sequentially digested at 37 1C for 2 h in collagenase/hyaluronidase, 0.05% Trypsin, DNAse I and Dispase (Stem Cell Technology, Tewksbury, MA, USA). The ensuing cell suspension was treated with red blood cell lysis buffer, rinsed with PBS and passed through a 40-um mesh after resuspension in Opti-Mem medium (Gibco, Grand Island, NY, USA). Cells were plated on gelatin-coated plates and grown in CnT-BM1 medium (Cell-N-Tec, Bern, Switzerland).

Mice

MMTV-ErbB2 mice harboring activated ErbB2 (strain FVBN-Tg(MMTV-ErbB2)NK1Mul/J) were from Jackson Labs (Bar Harbor, ME, USA). p53 R172H (called p53H/H) and control p53 null (p53-/-) mice (C57Bl6J background) were a gift from G. Lozano.11 p53 mice were interbred to generate H/- mice. Compound p53H/-;ErbB2 mice were generated by crossing ErbB2 into the p53-/- background and then breeding

the p53+/-;ErbB2 progeny with p53H/H animals. H/;ErbB2 mice were then crossed to generate p53H/H;ErbB2 and p53-/-;ErbB2 females for analysis. These F2 mice were of mixed background. Littermates were used for all analyses. Mice were treated according to the guidelines approved by the Institutional Animal Care and Use Committee.

Tissue microarrays

Tissue arrays of breast invasive ductal carcinomas (75 cases/150 cores; BR1504, Biomax, Rockville, MD, USA) with known TNM, pathologic grade and markers (ER, PR, Her2 and Ki67) were stained with antibodies to p53 (DO-1, Santa Cruz Biotechnology) or p326-HSF1 (Epitomics, Burlingame, CA, USA). Staining intensity was scored blindly as absent, weak, moderate, strong or very strong.

Statistics

Histoscores were analyzed using Spearman's rank correlation. Significance was calculated using Fisher's exact test.

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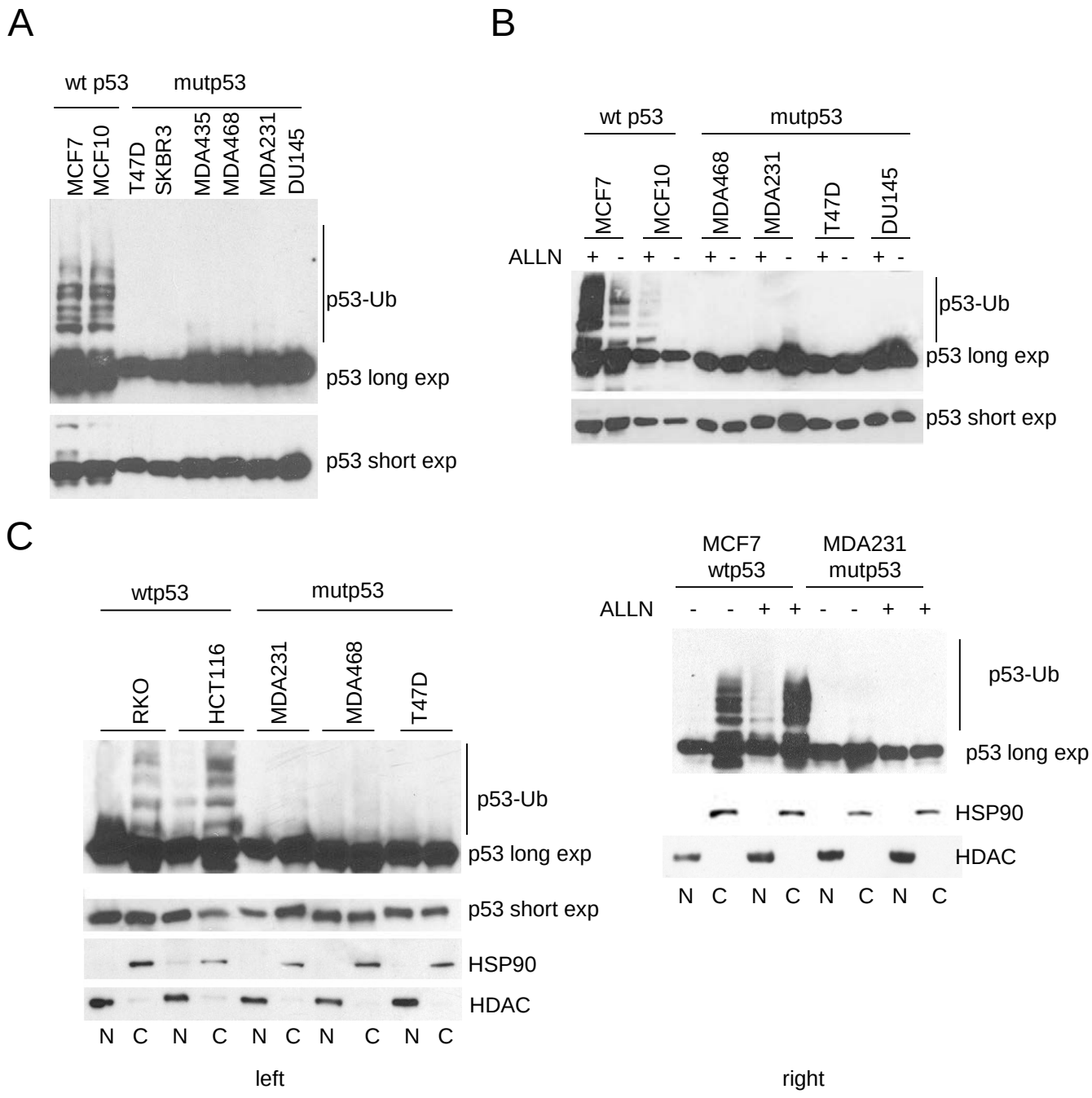
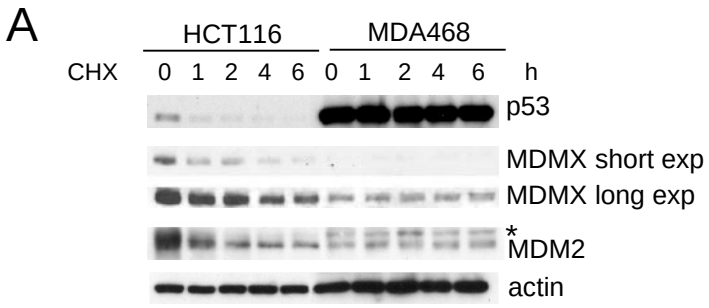


Figure 1. Tumor-derived mutant p53 proteins show a complete lack of ubiquitination, causing their profound degradation defect. (A) Ubiquitination of endogenous p53 in a panel of wild-type and mutant human cancer cell lines. p53 loading was normalized for comparable amounts of non-ubiquitinated p53. DO1 immunoblot. (B) Proteasome inhibition (by ALLN) results in accumulation of ubiquitinated p53 only in wtp53 cells, while mutp53 proteins remain completely non-ubiquitinated. Normalized loading and blotting as in A. (C) Left, Ubiquitinated wtp53 is mainly located in the cytoplasm. In contrast, both cytoplasm and nucleus are completely devoid of ubiquitinated mutp53. Right, Ubiquitinated wtp53 resides mainly in the cytoplasm and is stabilized by ALLN-mediated proteasome inhibition. In contrast, mutp53 is non-ubiquitinated in both compartments and remains so even after ALLN treatment. Immunoblots normalized for p53 loading. HDAC and Hsp90 as nuclear and cytoplasmic markers.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*
MDM2
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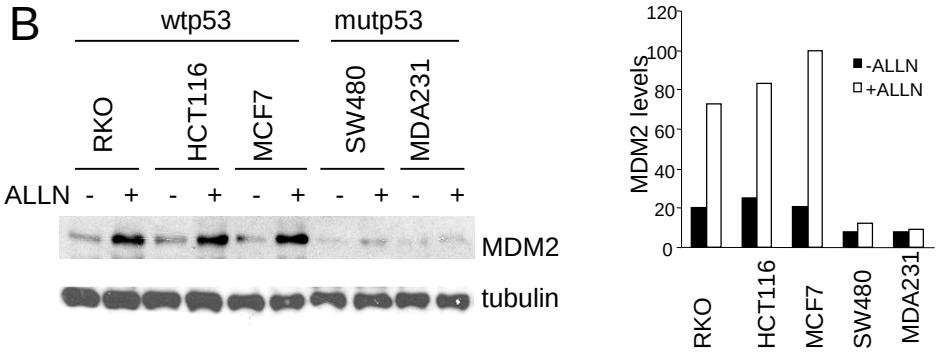
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mutp53
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MCF7
SW480
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ALLN - + - + - + - + - +
MDM2
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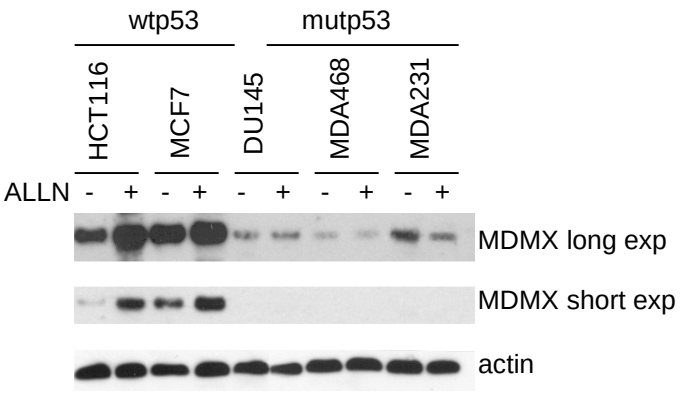
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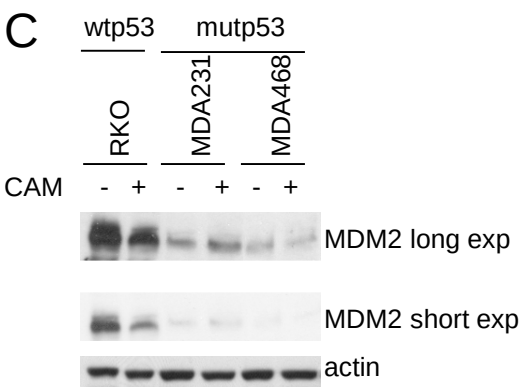
actin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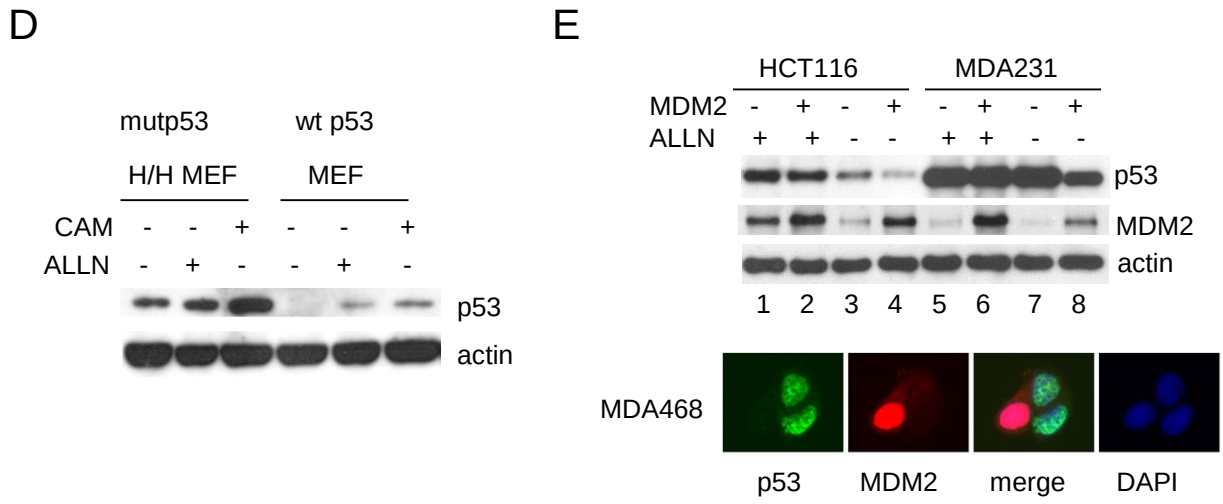


Figure 2. Selective impairment of MDM2 E3 ligase activity in mutp53 cancer cells. (A) Bona fide physiologic substrates of MDM2 (p53, MDMX and MDM2 itself) exhibit degradation deficiencies in mutp53 (MDA468) compared to wtp53 (HCT116) cells. Cycloheximide (CHX) chase for the indicated times. Actin, loading control. * denotes a nonspecific band. (B) Non-responsiveness of MDM2 and MDMX to proteasome inhibition and DNA damage in mutp53 cancer cells indicates selective impairment of the MDM2 E3 ligase activity in mutp53 cancer cells. Left,MDM2 activity is selectively impaired in mutp53 cancer cells. While wtp53 cells stabilized MDM2 more than 3-fold upon ALLN, no MDM2 stabilization occurred in mutp53 cells. Right, Corresponding densitometry quantitation of relative MDM2 levels is shown. Bottom, In contrast to wtp53 cells, MDMX levels failed to stabilize in mutp53 cells in response to ALLN. Cells treated with 25 μM ALLN for 12 h followed by immunoblotting. (C) DNA damage destabilizes MDM2 due to autoubiquitination in wtp53 but not in mutp53 cancer cells. Camptothecin (5 μM) for 2h. Immunoblot. (D) Normal mutp53 harboring p53H/H MEFs (which express somewhat higher constitutive levels in culture than wtp53 MEFs), properly stabilize mutp53 in response to genotoxic stress and proteasome inhibition. Immunoblots of cells treated with 5 μM Camptothecin or 25 μM ALLN for 6h. (E) Endogenous mutant p53 is readily degraded by overexpressed levels of ectopic MDM2. Top, immunoblots after transient transfection of MDM2 with and without 25 μM ALLN for 6h. Bottom, immunofluorescence of mutp53 MDA468 cells transfected with MDM2 (red). The transfected cell does not exhibit p53 staining (green). A-E Immunoblots, actin and tubulin as loading controls.

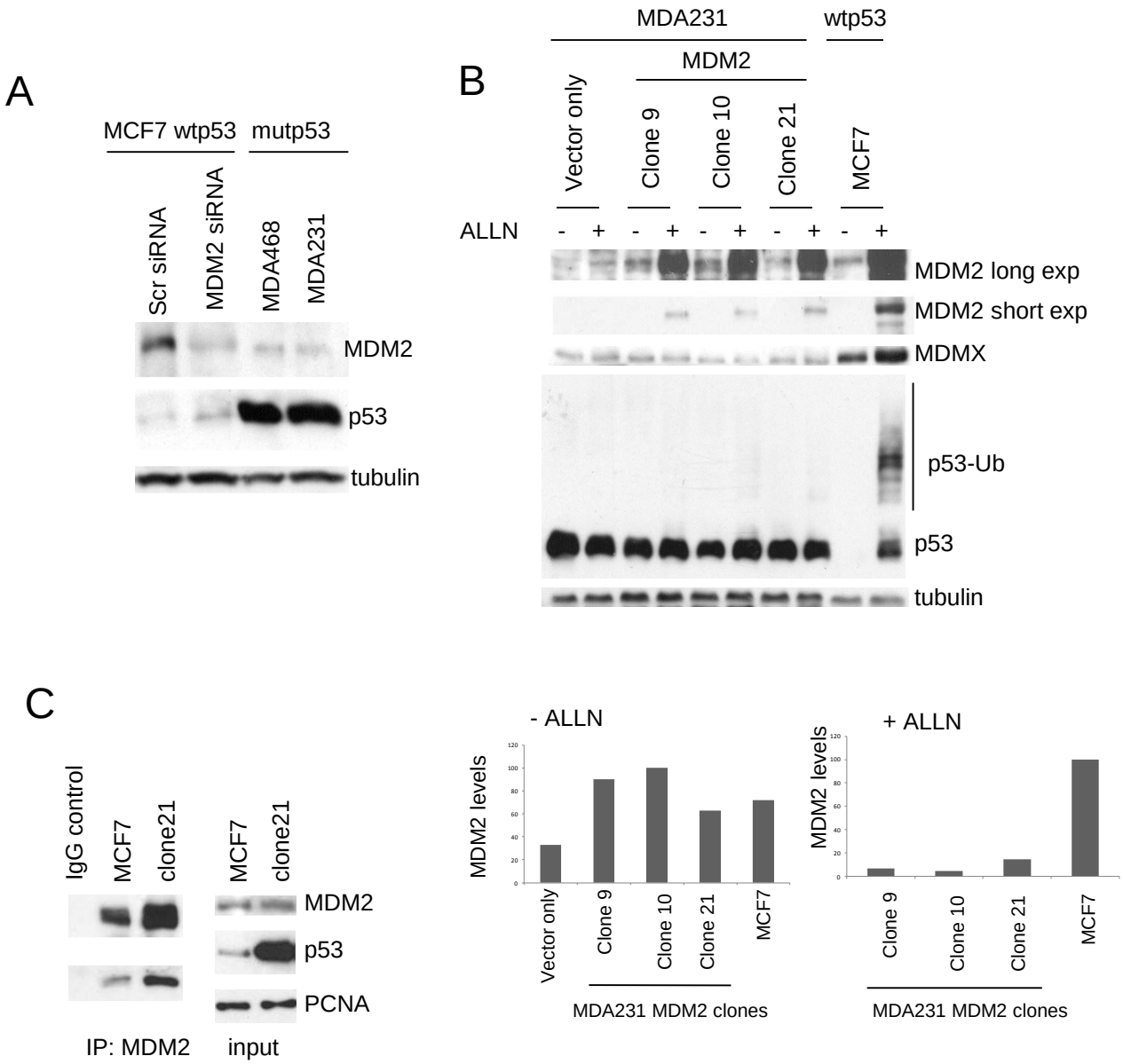
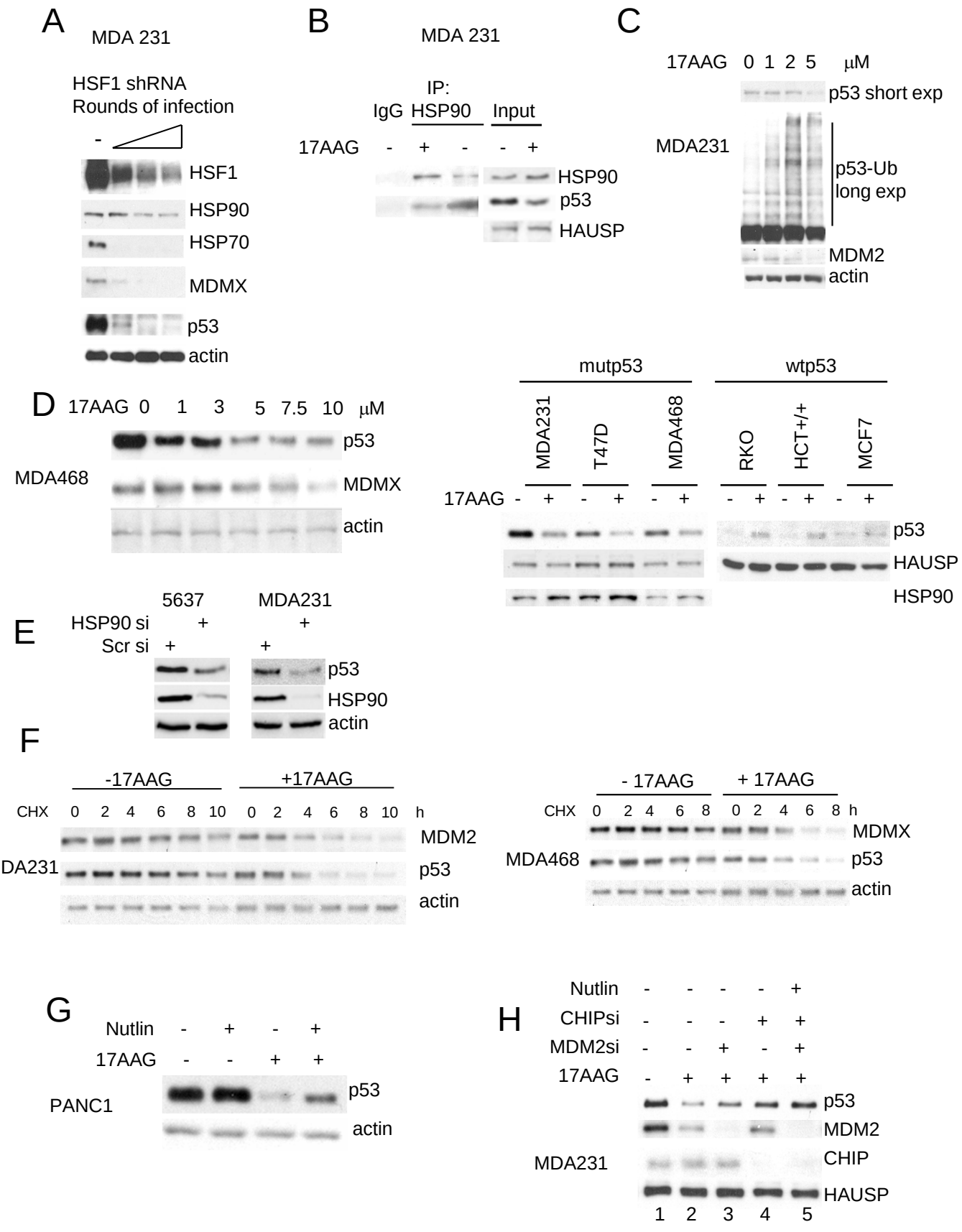


Figure 3. MDM2 activity is functionally impaired in mutant p53 cancer cells (A, B) Decreased levels of MDM2 are not the main determinant of mutp53 stability in cancer cells. A. MDM2 levels in wtp53 breast cancer cells (MCF7) were downregulated by siRNA to match those of mutp53 cells (MDA231, MDA468). However, knockdown of MDM2 in MCF7 cells elevates wtp53 levels only by less than 2-fold, and does not reach the 20-fold stabilization present in mutp53 cells. (B) Conversely, the lower MDM2 levels in mutp53 MDA231 cells were corrected back to those of wtp53 MCF7 cells. Stable MDM2 clones express 2- fold higher MDM2 levels. However, in all established MDA231 clones, mutp53 levels remained unaffected and ubiquitination non-detectable, even after challenge with ALLN (3 independent clones of 7 shown). Cells treated with 25 μM ALLN for 12h.MDM2 and MDMX display the same (self)-degradation defect as seen in Fig. 3, in contrast to wtp53 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 mutp53. MDM2 immunoprecipitation from MDA231-MDM2 clone 21 or wtp53 MCF7 cells, followed by immunoblots. PCNA, loading control.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Rounds of infection
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HSF1
HSP90
HSP70
MDMX
p53
actin
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MDA 231
IP:
IgG HSP90 Input
17AAG - + - - +
HSP90
p53
HAUSP
C
17AAG 0 1 2 5 μM
p53 short exp
MDA231
p53-Ub
long exp
MDM2
actin
D
17AAG 0 1 3 5 7.5 10 μM
MDA468
p53
MDMX
actin
mutp53
wtp53
MDA231
T47D
MDA468
RKO
HCT+/+
MCF7
17AAG - + - + - + - + - + - +
p53
HAUSP
HSP90
E
5637
MDA231
HSP90 si + +
Scr si + +
p53
HSP90
actin
F
-17AAG
+17AAG
CHX 0 2 4 6 8 10 0 2 4 6 8 10 h
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MDA231
p53
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+ 17AAG
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G
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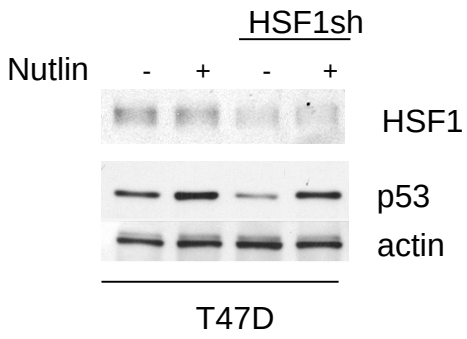

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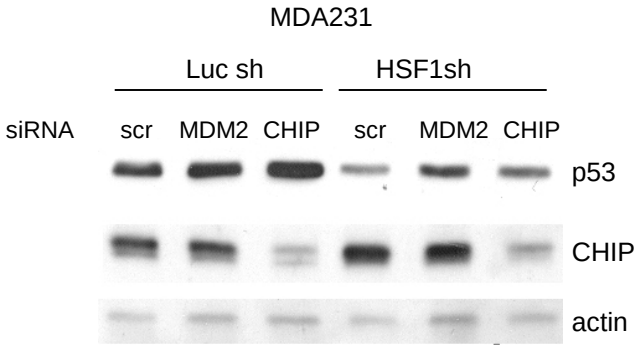
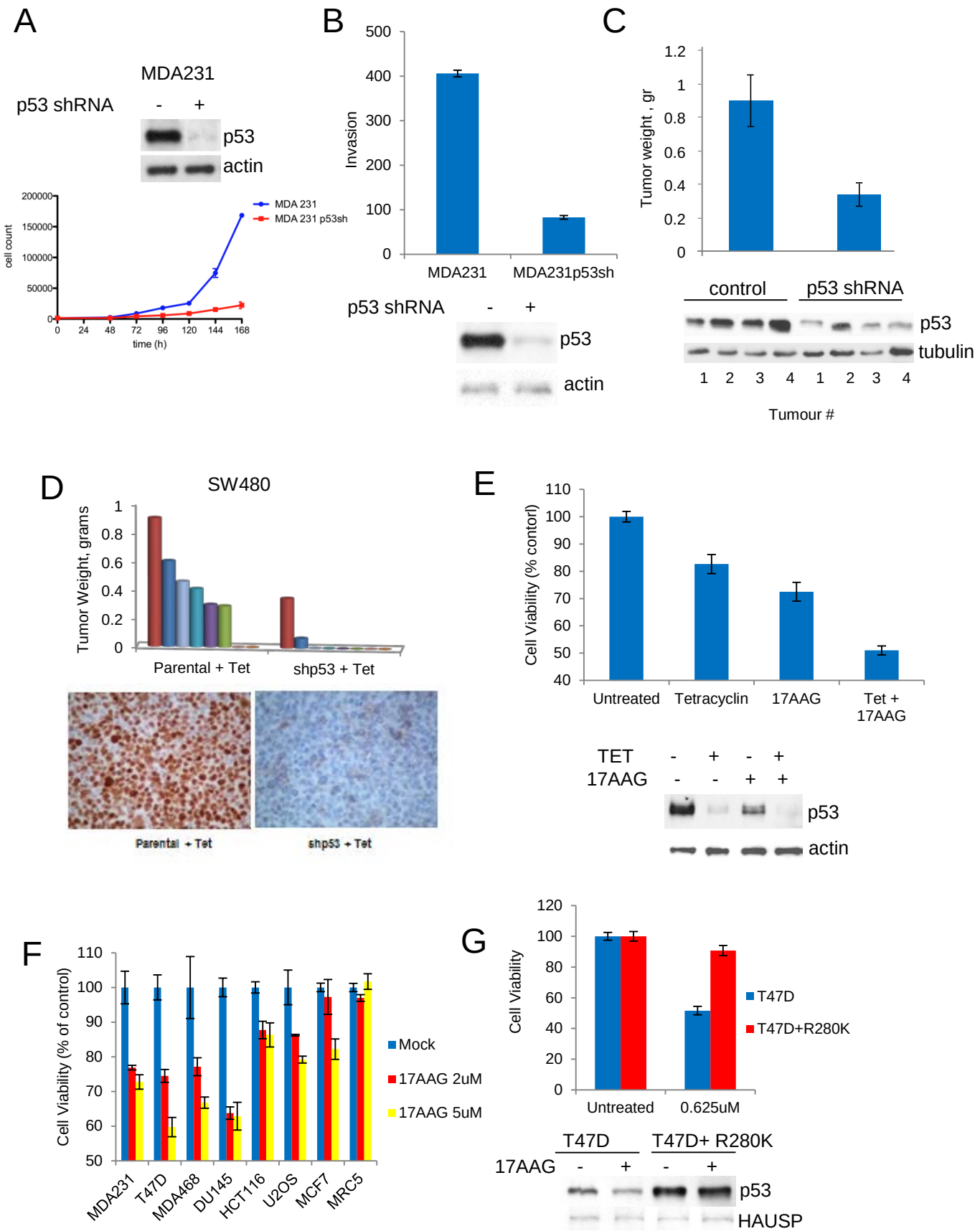


Figure 4. Stabilization of mutp53 in cancer cells is caused by the HSP90 chaperone machinerythat inhibits the MDM2 and CHIP E3 ligase activity. (A) HSF1 knockdown, which leads to downregulation of Hsp90 and Hsp70 proteins, induces destabilization of mutp53. The eukaryotic genome contains separate genes encoding constitutively expressed and inducible Hsp90. Only inducible Hsp90 transcription is controlled by HSF1.Note that the Hsp90 antibody recognizes both constitutive and inducible Hsp90, explaining the only partial obliteration of Hsp90 after HSF1 knockdown. (B) Hsp90 interference by17AAG causes release of mutp53 from the Hsp90 complex. Hsp90 (or IgG control) immunoprecipitation (1 mg protein each) from MDA231 cells before and after 17AAG treatment (5 μM for 24 h). HAUSP, loading control. (C) 17AAG induces ubiquitination of mutp53 in a dose-depended manner. Cells were treated with the indicated concentrations of 17AAG for 24h. (D) Left, 17AAG induces degradation of mutant p53 (and MdmX) in a dose dependent manner. 177AAG induces degradation of mutant but not wild-type p53 in cancer cells. Right Cells treated as indicated for 24h; 2 μg of mutp53 and 20 μg of wtp53 cell extracts probed. 17AAG induces degradation of mutp53 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 heat shock proteins, including Hsp90. This explains the slight increase in Hsp90. HAUSP, loading control. (E) Downregulation of Hsp90 protein by siRNA destabilizes mutp53 in MDA231 and 5637 cancer cells. Scr si, scrambled siRNA. Three days post transfection, cells were harvested and analyzed by immunoblots. Actin, loading control. (F) 17AAG decreases the half-life of mutp53, Mdm2 and MdmX. Cycloheximide chase (CHX) of MDA231 and MDA468. Cells treated with 5 μM 17AAG for 16h, then 17AAG plus 50 μg/ml CHX for the indicated times. (G) Nutlin partially prevents 17AAG-induced destabilization of mutp53, indicating MDM2 reactivation upon 17AAG. PANC1 cells harboring mutp53 were treated with 5 μM 17AAG with or without 10 μM Nutlin for 16h. Immunoblot. Actin, loading control. (H) siRNA-mediated knockdown of MDM2 and CHIP rescue 17AAG-induced destabilization of mutp53. (I) Nutlin prevents HSF1 knockdowninduced destabilization of mutp53, indicating MDM2 reactivation upon HSP90 downregulation. (J) HSF1 knockdown-mediated destabilization of mutp53 (lane 4) is partially reversed by concomitant siRNA-mediated knockdown of MDM2 or CHIP E3 ubiquitin ligases (lanes 5 and 6), indicating reactivation of both enzymes upon HSF1 downregulation.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p53
actin
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time (h)
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MDA 231 p53sh
B
Invasion
MDA231
MDA231p53sh
p53 shRNA - +
p53
actin
C
Tumor weight , gr
control
p53 shRNA
p53
tubulin
1 2 3 4 1 2 3 4
Tumour #
D
SW480
Tumor Weight, grams
Parental + Tet
shp53 + Tet
Parental + Tet
shp53 + Tet
E
Cell Viability (% contorl)
Untreated
Tetracyclin
17AAG
Tet + 17AAG
TET - + - +
17AAG - - + +
p53
actin
F
Cell Viability (% of control)
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T47D
MDA468
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17AAG 2uM
17AAG 5uM
G
Cell Viability
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p53
HAUSP

Fig. 5

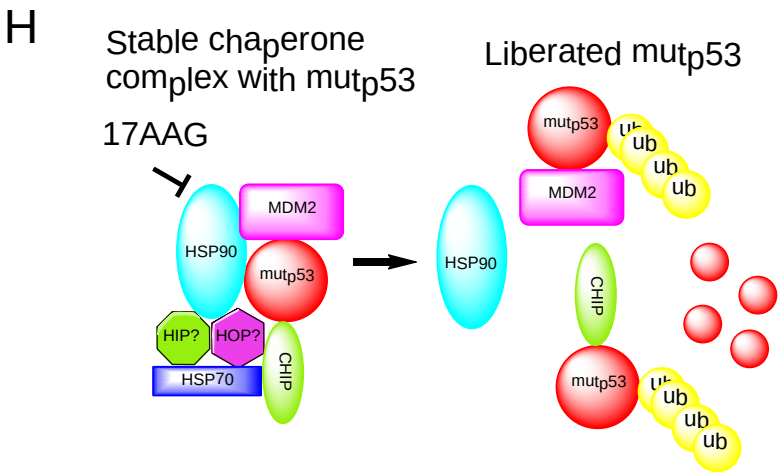
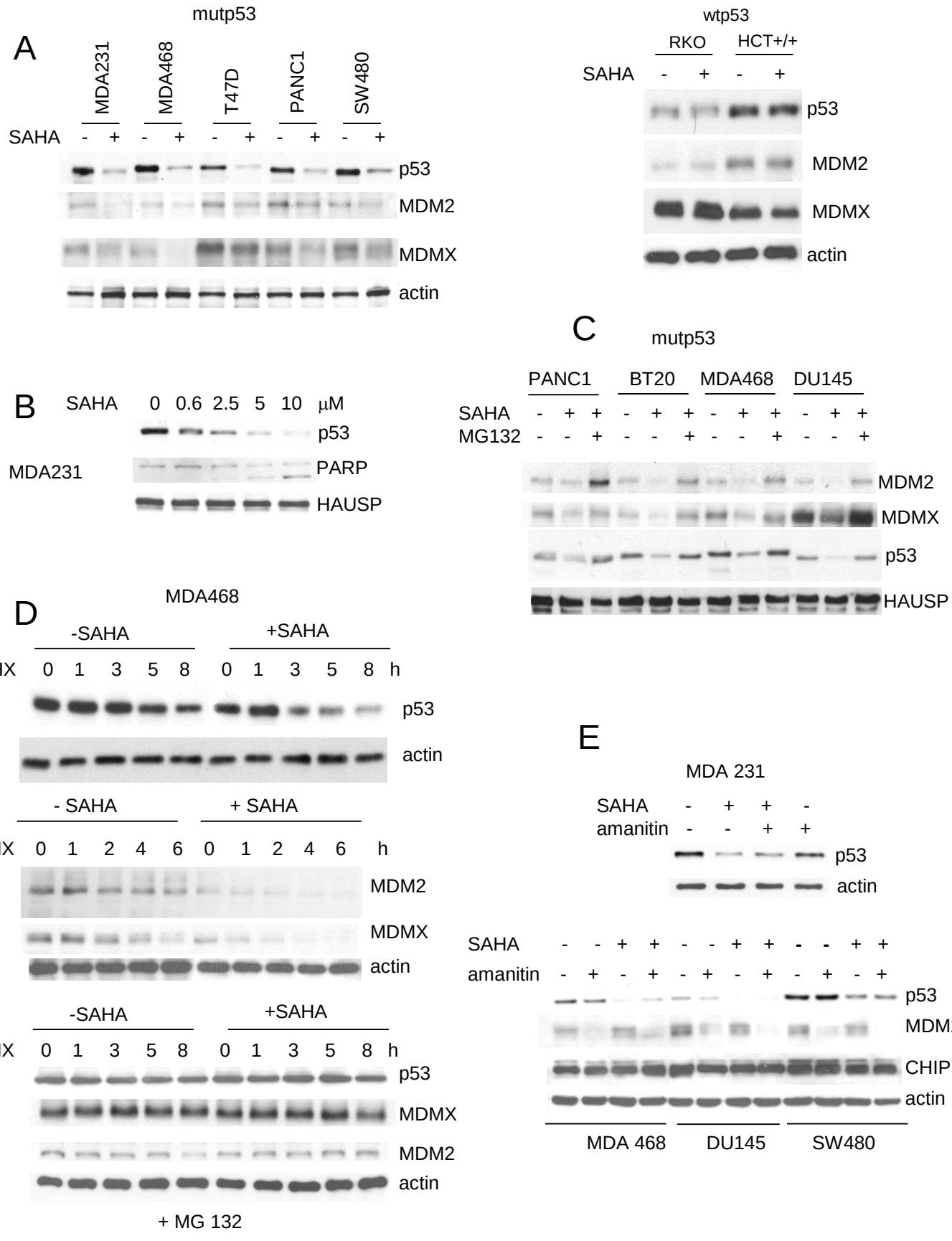


Figure 5. 17AAG reduces cell viability more profoundly in mutp53 compared to wtp53 cancer cells. (A-D) Knockdown of mutant p53 by shRNA inhibits proliferation and invasion of human cancer cells in vitro and in vivo. shp53-mediated stable knockdown of mutp53 in MDA231 compared to parentals, assayed for proliferation in vitro (A) and in tumor xenografts in nude mice (C, D). (C) Average tumors weight (n=12 each) at day 20. Bars represent mean +/- standard error. All tumors show significant reduction of mutp53 levels (examples shown). (B) Knockdown of mutp53 in MDA231 cells by Tet-inducible shp53 inhibits their invasion. Matrigel Boyden chambers and immunoblot (bottom). (D) Knockdown of mutp53 in SW480 colon cancer cells by Tet-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 mutp53 by 17AAG (2 μM) and/or Tetracycline-inducible shp53 RNAi decreases cell viability of mutp53 SW480 cells proportional to the extent of mutp53 destabilization (bottom).CTB assay. Cells treated with Tetracyclin for 24h or left untreated, then concurrently with empty vehicle and/or 2 μM 17AAG for 24h. (F) 17AAG inhibits cell viability more profoundly in mutp53 than in wtp53 cancer cells. CTB assay. Cells treated with 2 or 5 μM 17AAG for 24h or left untreated. (G) 17AAG loses killing efficacy when its ability to degrade mutp53 is overwhelmed by excess ectopic mutant p53. The indicated T47D cells were treated with 0.625 μM 17AAG or mock-treated for 48h. Top, Cell viability assay (CTB). Bottom, Corresponding immunoblot of empty vector or mutp53 R280K overexpressing T47D cells. HAUSP, loading control. (H) Proposed model of regulation of mutp53 stability by the HSP90 multi-chaperone machinery in cancer cells. See text for explanation.

Fig. 5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RKO
HCT+/+
SAHA - + - +
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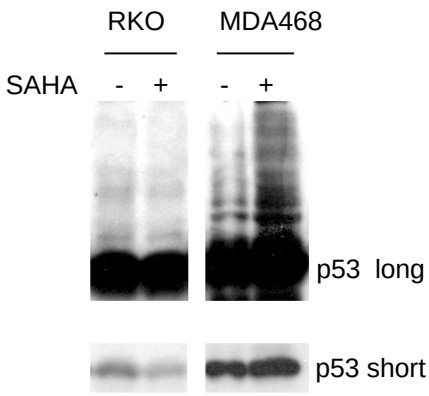


Figure 6. SAHA downregulates mutp53 but not wtp53. This effect occurs at the level of protein degradation but not at the level of transcription. (A) SAHA (5 uM) strongly downregulates mutant p53, MDM2 and MDMX protein levels. In contrast, SAHA does not alter levels of wild-type p53 protein. A panel of human tumor cell lines harboring mutant or wild-type p53 were analyzed by immunoblot as indicated. Actin,loading control. (B) SAHA-mediated downregulation of mutant p53 is dose-dependent and correlates with induction of PARP cleavage. (C) Proteosome inhibitor MG132 (5 uM) rescues SAHA mediated downregulation of mutp53, MDM2 and MDMX. Cells were simultaneously treated with SAHA (5 uM) and MG132 (5 uM) for 16h. HAUSP as a loading control. (D) SAHA (5 uM) dramatically decreases the half-life of mutant p53, MDM2 and MDMX, all bona fide physiologic substrates of MDM2. Cycloheximide (CHX) chase for the indicated times. Actin, loading control. Bottom, proteasome inhibitor MG132 (5 uM) rescues SAHA induced destabilization of mutp53, MDM2 and MDMX after cycloheximide treatment. (E) The SAHA-mediated downregulation of mutant p53 does not occur at the level of transcription. Mutp53 cells were treated with SAHA (5 uM) and/or α-amanitin (10 µg/ml) for 16h, a potent and specific transcriptional inhibitor of RNA polymerase II. (F) SAHA induces ubiquitination of mutant but not wild-type p53. RKO (wtp53) and MDA468 (mutp53) cells were grown in the presence or absence of 5 µM SAHA. p53 loading was normalized for comparable amounts of non-ubiquitinated p53. DO1 immunoblot.

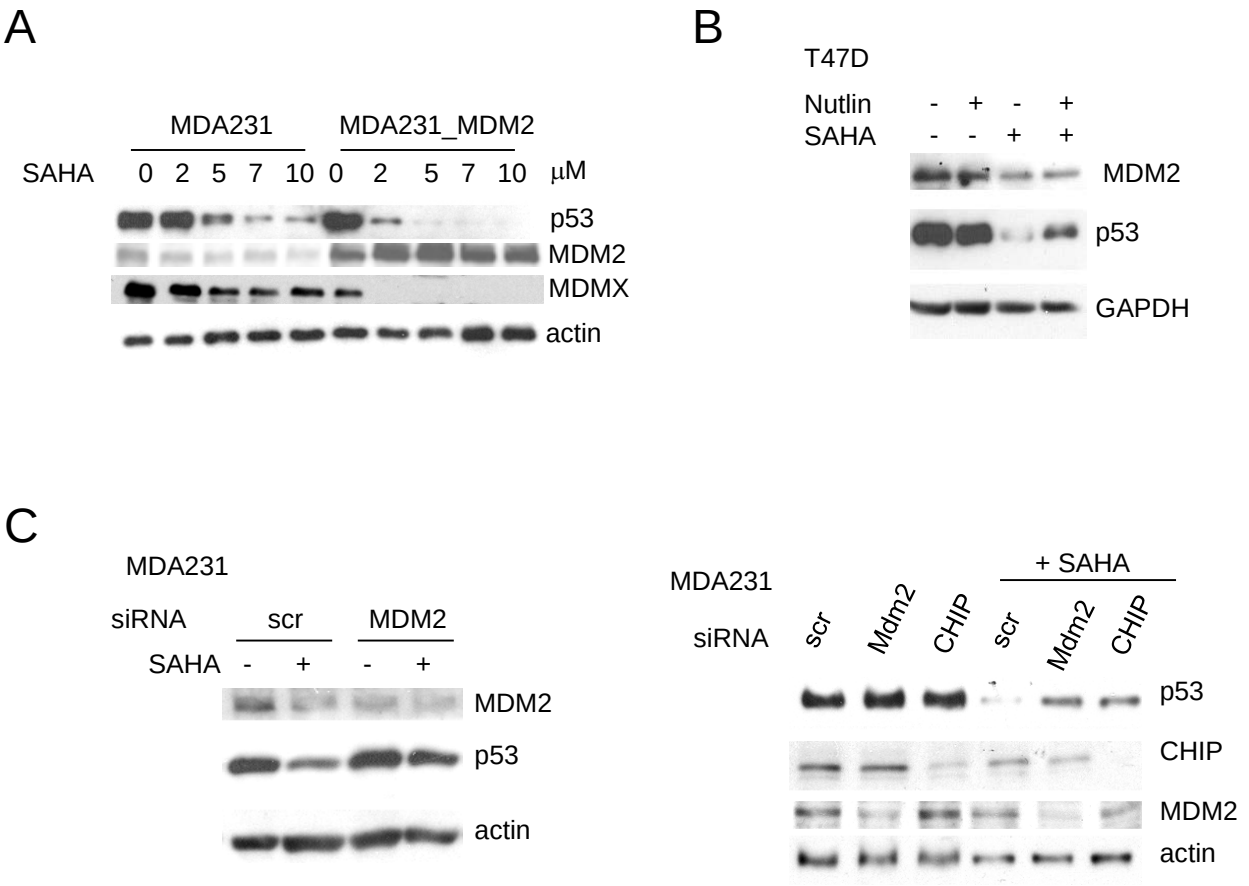


Figure 7. SAHA-induced degradation of mutp53 is mediated by reactivation of MDM2 and CHIP E3 ligases. (A) Overexpression of MDM2 enhances SAHA-induced degradation of mutp53 and MDMX. The cell system used here is described by Li et al 7 where we show that in human cancer cells endogenous mutant p53 - despite its ability to interact with MDM2 - suffers from a profound lack of ubiquitination as the root cause of its degradation defect. Multiple lines of evidence indicate the functional impairment of MDM2 in mutp53 cancer cells by the HSP90 chaperone. We found that in contrast to transiently overexpressed MDM2, physiologically tolerated, stably overexpressed MDM2 is silent and fails to affect ubiquitination and mutp53 levels, indicating the presence of selective pressure against active MDM2 in mutp53 cancer cells. Immunoblot of parental MDA231 and cells stably overexpressing MDM2. Actin as loading control. (B) Nutlin partially prevents SAHA-induced destabilization of mutp53, indicating MDM2 reactivation upon SAHA (5 uM). GAPDH, loading control. (C) siRNA-mediated knockdown of MDM2 and CHIP partially rescues SAHA-induced (5 uM) destabilization of mutp53.

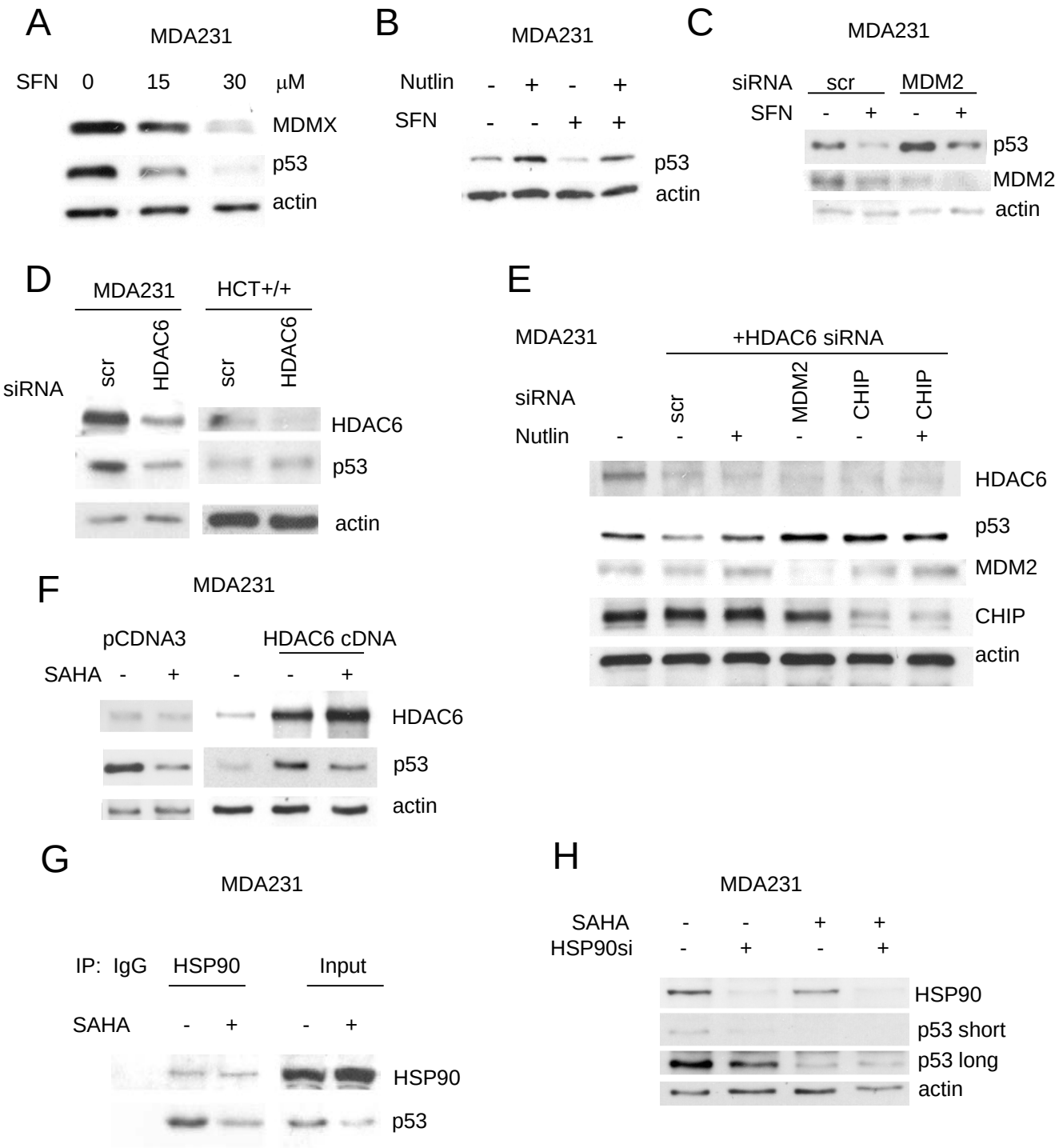
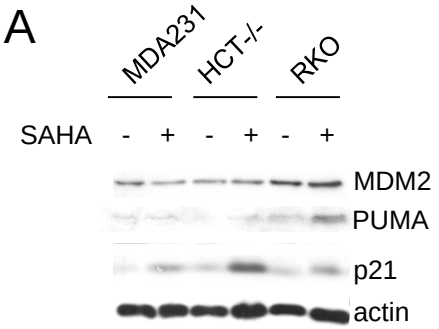
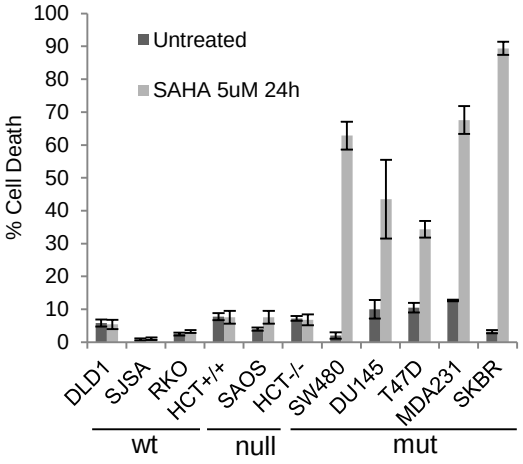


Figure 8. Inhibition of HDAC6 inhibits HSP90 and destabilizes mutp53 by reactivating MDM2 and CHIP. (A) HDAC6 inhibitor Sulfaraphan (SFN) destabilizes mutp53 and MDMX. Nutlin (B) and MDM2 siRNA (C) partially prevent SFN-induced degradation of mutp53. (D) Likewise, HDAC6 inhibition by siRNA also destabilizes mutp53 (in MDA231) but does not destabilize wtp53 (in HCT116). (E) siRNA-mediated knockdown of HDAC6 results in degradation of mutp53 that is rescued by Nutlin (lane 3) or by siRNA against MDM2 and CHIP. Scr scrambled siRNA. Immunoblot. (F) Conversely, HDAC6 overexpression stabilizes mutp53, which is significantly rescued by concomitant SAHA. Immunoblot. (G) SAHA (5 uM) inhibits the complex between Hsp90 and mutp53. Lysates from MDA231 cells left untreated or treated with SAHA were immunoprecipitated with IgG or anti-Hsp90. (H) In HSP90-ablated cells, SAHA does not induce further destabilization of mutp53. MDA231 cells were transfected with siHSP90 or scrambled siRNA control. After 24h, cells were treated with 2.5 uM SAHA for an additional 24h followed by immunoblot.





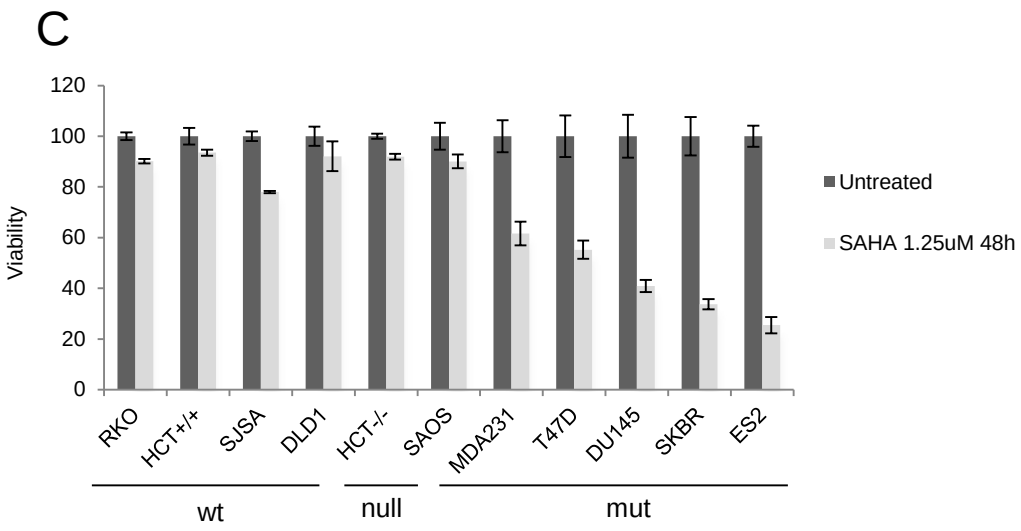


Table 1. IC50 of SAHA

Cell Line	IC50 (μM)	p53 status
RKO	393.0	wt
HCT+/+	128.0	wt
T47D	1.732	mut
MDA231	1.105	mut
ES2	1.980	mut

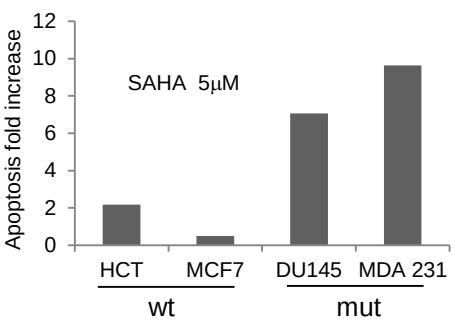


Fig. 9

E

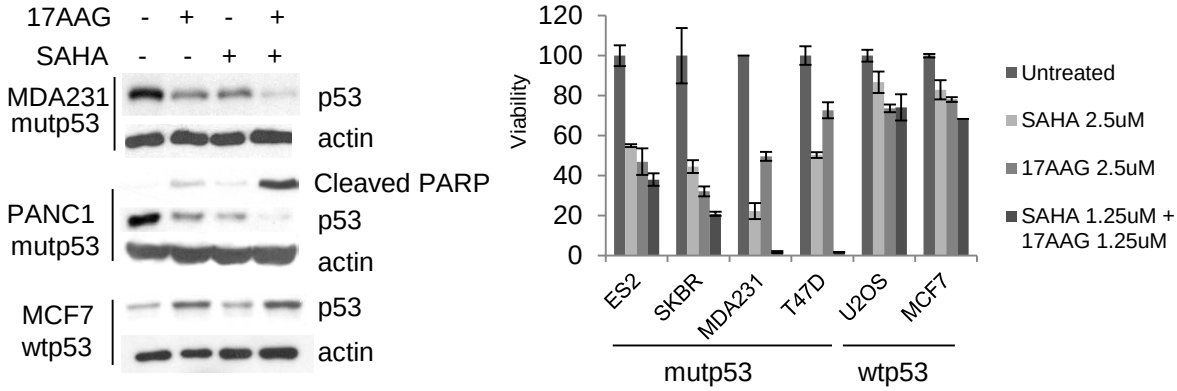
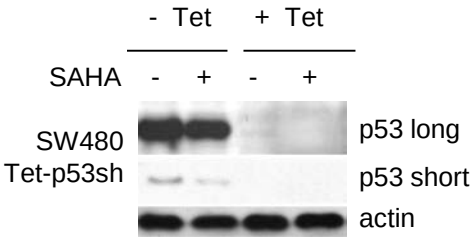
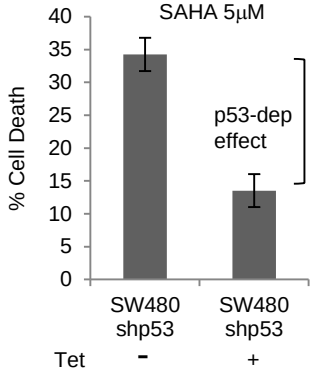


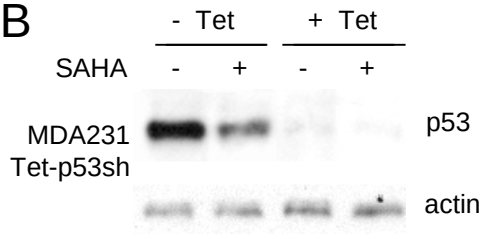
Figure 4. SAHA shows preferential cytotoxicity for mutp53 tumor cells. (A) SAHA does not pharmacologically rescue mutant p53 to assume wild type function. MDM2 is not induced. Although p21 is slightly induced, it appears to be in a p53-independent manner, based on p53-deficient HCT116 cells (HCT-/-). SAHA was used at 5 uM. (B-D) SAHA shows strong cytotoxic cell killing towards mutp53 tumor cells, as measured by trypan blue exclusion assay (B), cell viability CTB assay (C), and subG1 FACS (D). In contrast, wtp53 and p53 null tumor cells show only a minimal cytotoxic response to SAHA. Random panel of human tumor cells. (E) SAHA and 17AAG can synergize to induce preferential apoptosis of mutp53 cancer cells. Co-treatment of SAHA and 17AAG causes a synergistic loss of cell viability specifically in MDA231 and T47D. Combined efficacy correlates with the degree of mutp53 destabilization and PARP cleavage (Right immunoblots). This synergism is due to complementary drug targets within the HSP90 chaperone machinery.

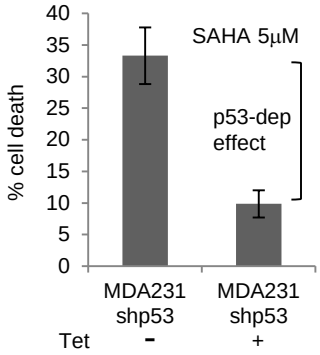
A

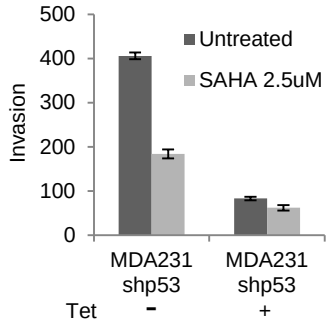

- Tet
+ Tet
SAHA - + - +
SW480
Tet-p53sh
p53 long
p53 short
actin


SAHA 5μM
% Cell Death
40
35
30
25
20
15
10
5
0
p53-dep
effect
SW480
shp53
SW480
shp53
Tet
-
+

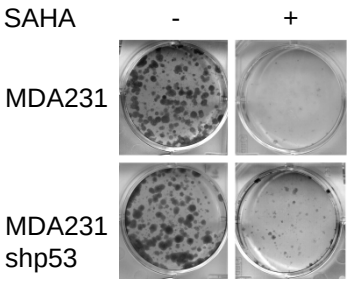
B

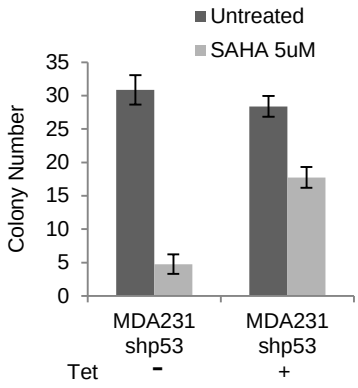

- Tet
+ Tet
SAHA - + - +
MDA231
Tet-p53sh
p53
actin


SAHA 5μM
% cell death
40
35
30
25
20
15
10
5
0
p53-dep
effect
MDA231
shp53
MDA231
shp53
Tet
-
+

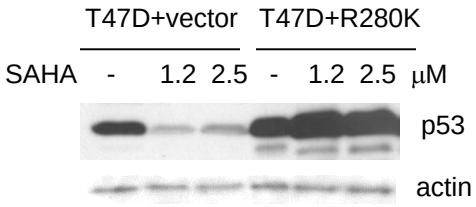

Untreated
SAHA 2.5uM
Invasion
500
400
300
200
100
0
MDA231
shp53
MDA231
shp53
Tet
-
+

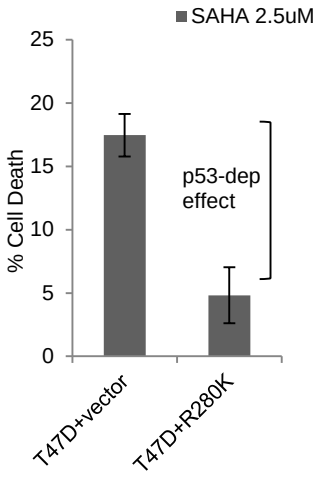
C


SAHA - +
MDA231
MDA231
shp53

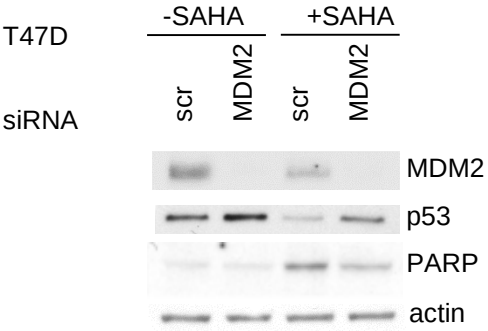

Untreated
SAHA 5uM
Colony Number
35
30
25
20
15
10
5
0
MDA231
shp53
MDA231
shp53
Tet
-
+

D

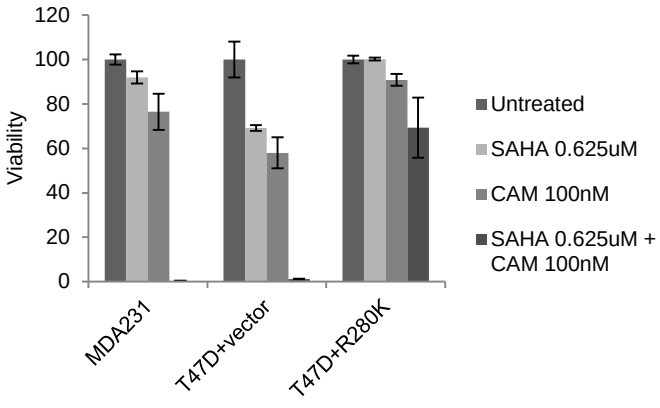

T47D+vector
T47D+R280K
SAHA - 1.2 2.5 - 1.2 2.5 μM
p53
actin

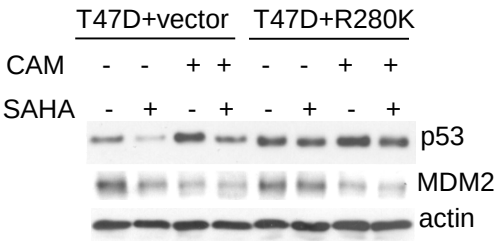

SAHA 2.5uM
% Cell Death
25
20
15
10
5
0
p53-dep effect
T47D+vector
T47D+R280K

E


T47D
siRNA
-SAHA
+SAHA
scr
MDM2
scr
MDM2
MDM2
p53
PARP
actin

F


Viability
120
100
80
60
40
20
0
MDA231
T47D+vector
T47D+R280K
Untreated
SAHA 0.625uM
CAM 100nM
SAHA 0.625uM + CAM 100nM


T47D+vector
T47D+R280K
CAM - - + + - - + +
SAHA - + - + - + - +
p53
MDM2
actin

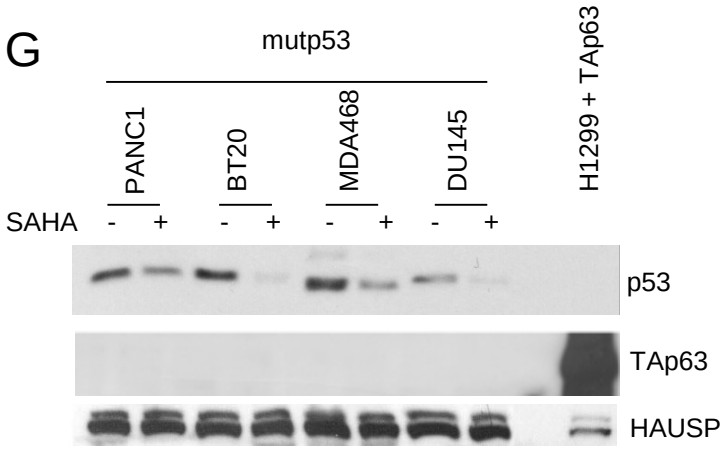


Figure 10. Causality - SAHA's preferential cytotoxic effect on mutp53-harboring cancer cells is due to a significant degree to its ability to degrade mutp53. (A-C) Pseudo-null lines were generated to eliminate mutp53 as a target of SAHA. Cells were pretreated with tetracycline to achieve 'isogenicity'. Cells with high (control) and very low levels of mutp53 were then treated with SAHA. In both MDA231 and SW480, SAHA partially loses cytotoxicity when mutp53 is very low. (A, B) In mutp53-harboring cancer cells SAHA loses over 50% killing efficacy when mutp53 is knocked down. Left, Immunoblots of (A) SW480 and (B) MDA231 cells stably harboring Tet-inducible shp53. Middle, Trypan Blue exclusion assays. Percent increase in cell death of SAHA-treated cells relative to their respective untreated controls without SAHA. (B, Right) Knockdown of mutp53 by SAHA or/and Tet- inducible shRNA inhibits invasion. (B, Left) Inhibition of invasion directly correlates with the extent of mutp53 destabilization. Aliquots were used for the Matrigel Boyden chambers and corresponding immunoblot. (C) Clonogenicity assay of MDA231 cells harboring Tet-inducible shp53 in response to SAHA. Right, Quantitation. For experiments in a-c, cells were pretreated with tetracycline for 48h and then seeded for subsequent SAHA treatment (5 uM) for an additional 24h. (D) Likewise, SAHA loses killing efficacy when its ability to degrade mutp53 is overwhelmed by excess ectopic mutant p53. Left, Immunoblots of empty vector or mutp53 R280K overexpressing T47D. Right, Increase in cell death of SAHA-treated cells relative to their respective untreated controls without SAHA. Trypan Blue exclusion assay. (E) siRNA-mediated knockdown of MDM2 partially rescues SAHA-induced destabilization of mutp53 and inhibits the cytotoxic effect of SAHA, indicated by reduced PARP cleavage. Cells were transfected with siMDM2 or scrambled siRNA, followed by SAHA treatment (5 µM) for 16h. Immunoblot, actin as loading control. (F) SAHA strongly chemosensitizes mutp53 cancer cells and this is due to its ability to degrade mutp53. Left Viability of MDA231, T47D vector and T47D cells overexpressing mutp53 R280K (see Figure 5d) after low dose camptothecin (100 nM) and SAHA (625 nM) treatment alone and in combination. CTB assays. Right Corresponding immunoblots of the T47D set. (G) SAHA fails to induce TAp63 protein in mutant p53 cancer cells. Despite multiple forced attempts (overloading, overexposure), we were unable to detect SAHA-induced TAp63 protein levels in any of the cell lines. Immunoblot with a pan-p63 antibody (H137) of total cell lysates (20 µg per lane) from mutp53 cancer cells grown in the absence or presence of SAHA (5 µM) for 24h. As control, 2 µg of total cell lysate from H1299 cells transfected with a TAp63α plasmid was loaded.

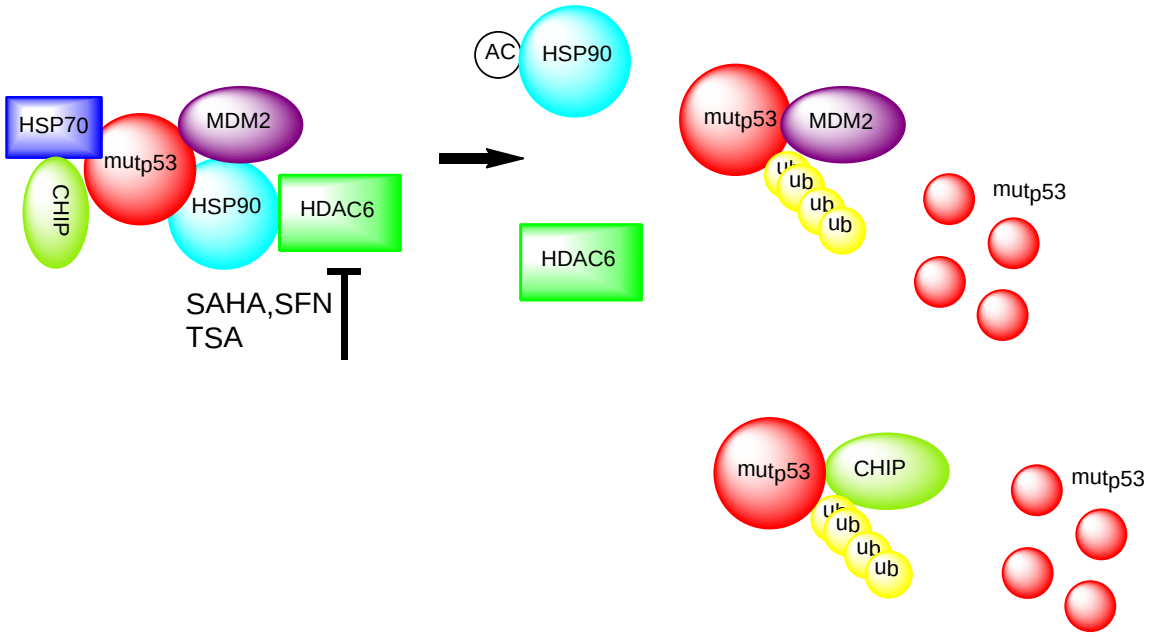


Figure 11. Proposed Model of SAHA-mediated destabilization of mutant p53 by inhibiting the HDAC6-Hsp90 chaperone axis. Based on results from us and others, we propose the following model. Normal tissues that harbor missense mutp53 are able to efficiently control their mutp53 levels despite the fact that their MDM2 levels are only supported by constitutive P1 promoter-derived transcription. In contrast, tumor-specific stabilization of mutp53 proteins, which contributes to driving the tumor phenotype, depends on a second alteration these cells undergo upon malignant transformation. This is their addiction to support from the activated heat shock machinery for survival. In contrast to wtp53, the aberrant conformation of mutp53 proteins requires permanent heat shock support, thus mutp53 is stably engaged in complexes with the highly activated HSP90 chaperone to prevent 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 mutp53's GOF. The E3 ligases MDM2 and CHIP, which in principle are capable of degrading mutp53, might also be trapped in this complex in an inactive state. Since mutp53 is fully competent to bind to MDM2, HSP90 likely binds to pre-existing mutp53-MDM2 complexes. Alternatively, chaperone-bound mutp53 could recruit MDM2. HDAC6 is a cytoplasmic non-histone HDAC that deacetylates Hsp90 and an obligate positive regulator of the HSP90 chaperone activity. Inhibiting HDAC6 by SAHA and related drugs leads to hyperacetylation and inhibition of Hsp90. This destroys the complex, releases mutp53 and enables MDM2/CHIP-mediated degradation.

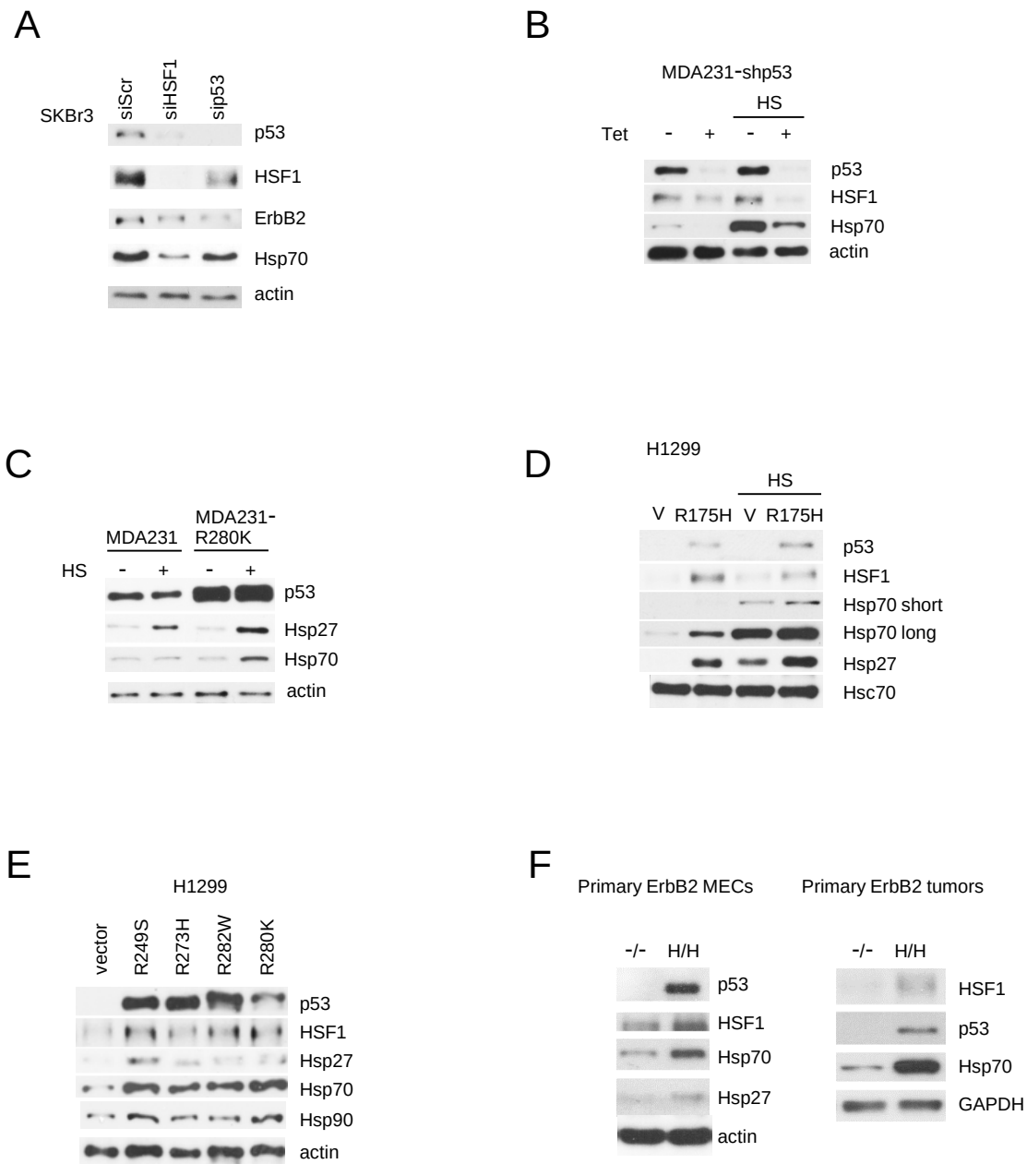


Figure 12. Mutant p53 upregulates HSF1 protein and augments the heat shock response (A) siRNA-mediated depletion of mutp53 in SKBr3 cells results in downregulation of HSF1 and its transcriptional target Hsp70. siHSF1 and scrambled siRNA as controls. (A-C, E-F) actin, loading control. (B) Tet-inducible p53 shRNA downregulates HSF1 and its target Hsp70, and is further enhanced by heat shock (HS, 43°C, 1h). Stable MDA231-shp53 cells. (C) Conversely, excess ectopic native mutp53 R280K in MDA231 cells further increases the HSF1 targets Hsp70/Hsp27 upon heat shock. (D) Ectopic mutp53 R175H in p53-null H1299 cells upregulates HSF1 and its targets Hsp70/Hsp27, which is further enhanced by heat shock. Hsc70, loading control. (E) Upregulation of HSF1 and its targets in response to all tested p53 mutants. (F) Compared to littermate p53-/-;ErbB2 tissues, both primary mammary epithelial cells (MECs, left) and mammary tumors (right) from p53H/H;ErbB2 mice show increased levels of HSF1 and its heat shock protein targets. Actin and GAPDH, loading controls.

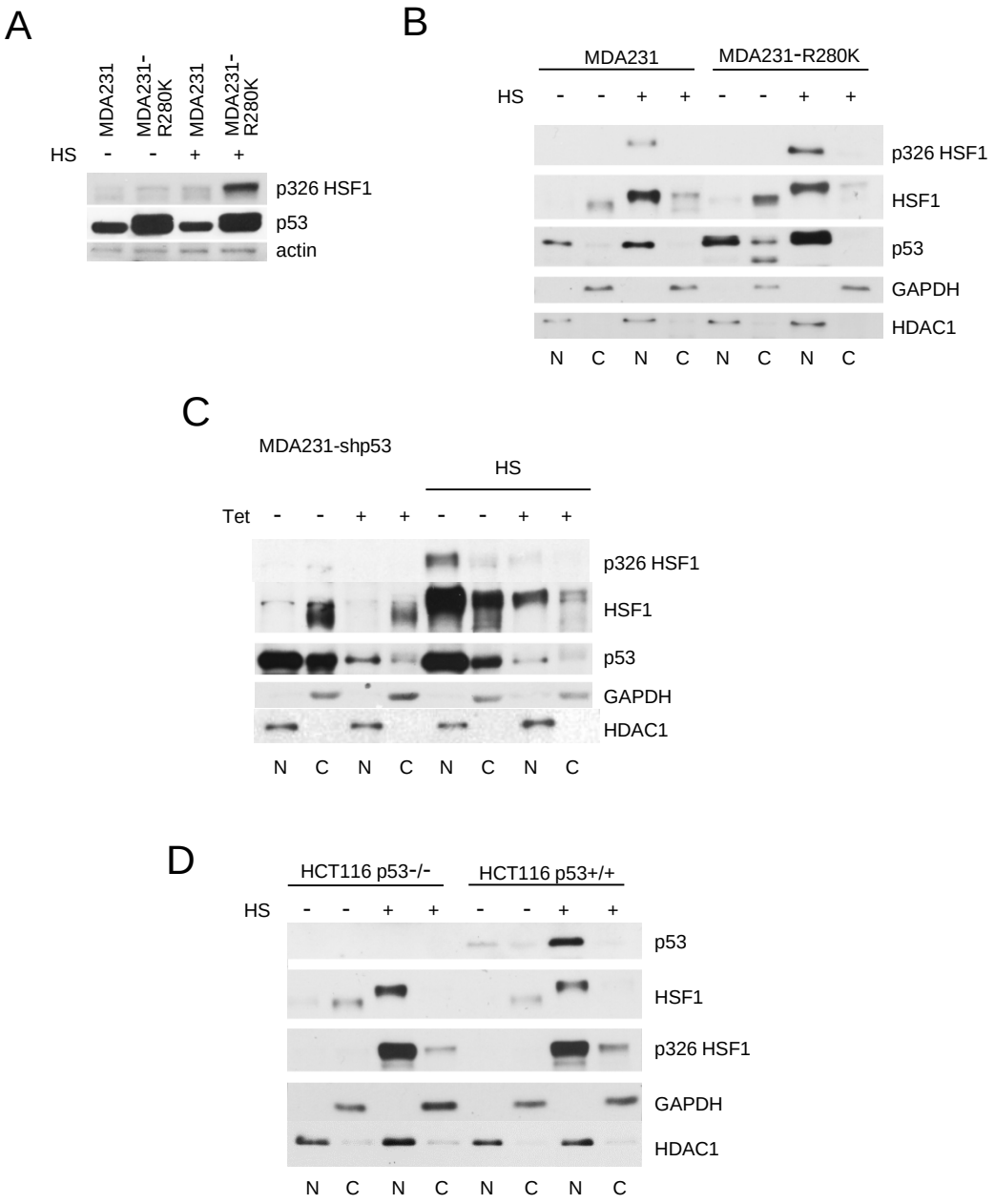


Figure 13. Mutant p53 promotes HSF1 activation via Ser326 phosphorylation (A) Activated p-Ser326 HSF1 is greatly upregulated in MDA231R280K cells after heat shock (HS, 43°C, 1h). Actin is loading control. (B) MDA231R280K cells induce higher levels of activated nuclear p-Ser326 HSF1 after heat shock than vector controls. The slower migration of total HSF1 detectable in 6% SDS PAAG upon HS is due to phosphorylation. (C) Tet-inducible p53 shRNA decreases total HSF1 and activated p-Ser326 HSF1 in the nuclear fraction of MDA231 cells after heat shock (43°C, 1h) compared to vehicle-treated control cells. The slower migration of total HSF1 detectable in 6% SDS PAAG upon HS is due to phosphorylation. (D) HSF1 is specifically regulated by mutant but not wildtype p53. No difference in HSF1 level or activation between HCT116 p53-/- vs p53+/+ cells +/- heat shock. The slower migration of total HSF1 detectable in 6% SDS PAAG upon HS is due to phosphorylation. (B-D) GAPDH and HDAC1 as cytoplasmic and nuclear markers, respectively.

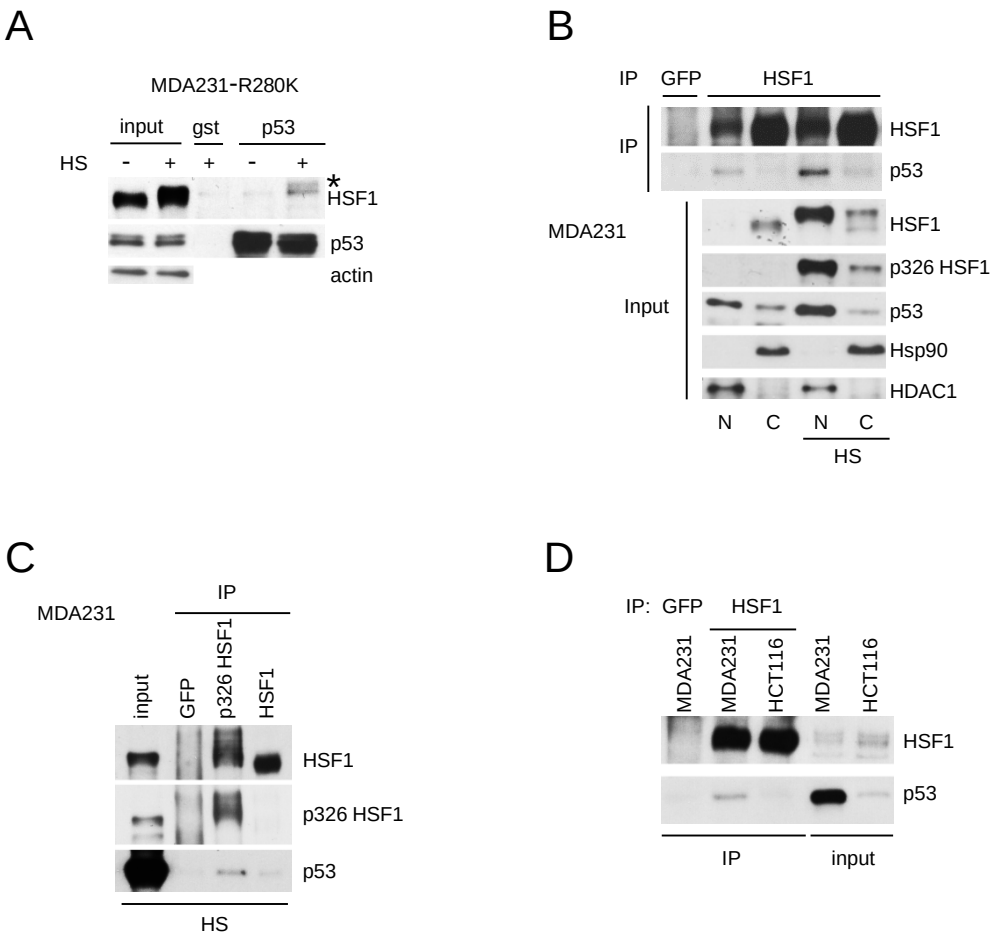
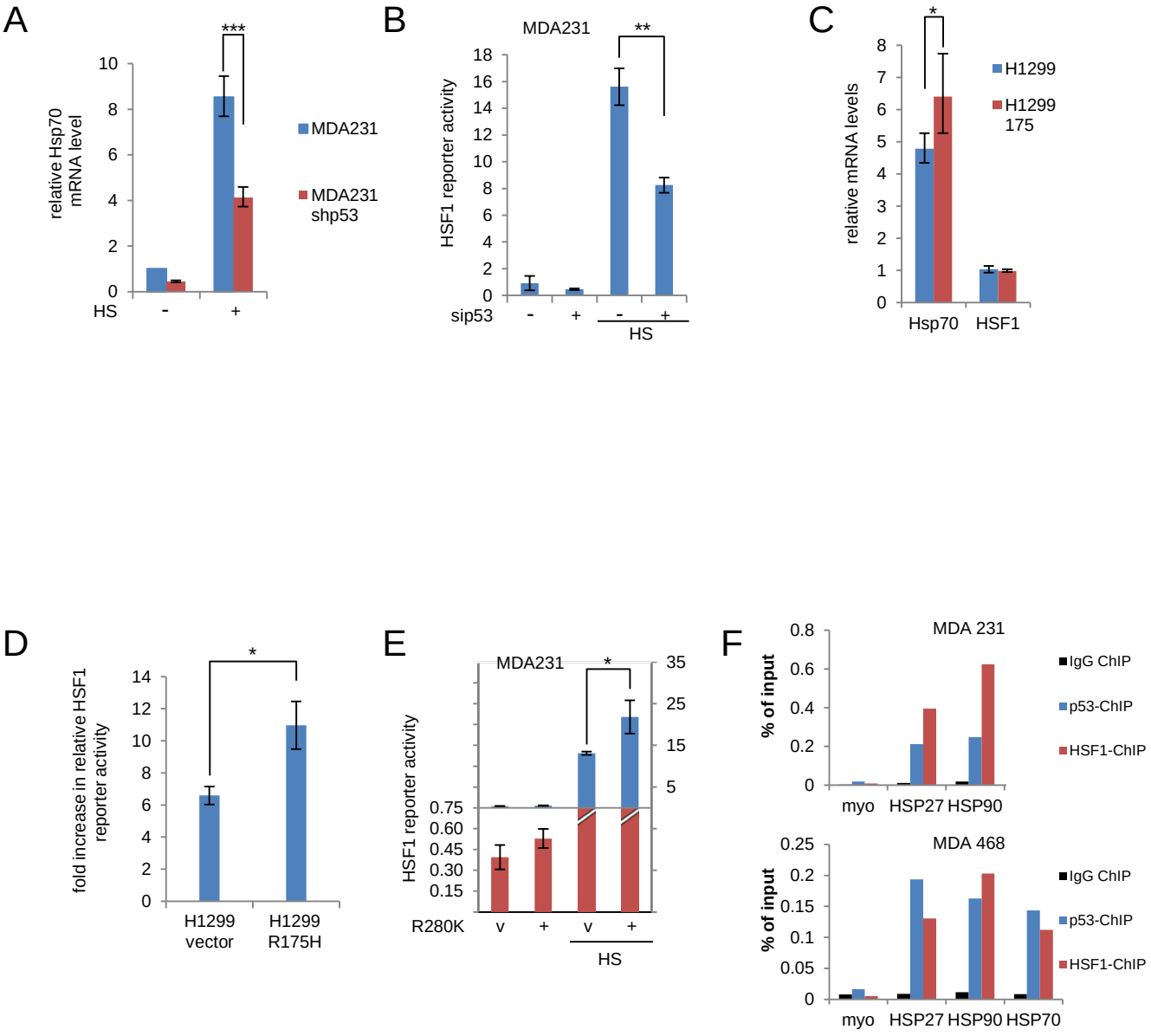


Figure 14. Mutant p53 interacts with activated HSF1 in the nucleus (A) mutp53-HSF1 complexes are induced by heat shock (HS, 43°C, 1h). Whole cell lysates of MDA231R280K cells were immunoprecipitated with p53 or irrelevant GST antibodies and immunoblotted for HSF1 and p53.
*-the slower migration band of total HSF1 detectable in 6% SDS PAAG corresponds to the phosphorylated form of HSF1. (B) Likewise, endogenous mutp53-HSF1 complex in the nucleus is induced by heat shock. Nuclear and cytoplasmic fractions from MDA231 cells -/+ HS were immunoprecipitated with HSF1 or irrelevant GFP antibodies and blotted for p53 and HSF1. HSP90 and HDAC1 as cytoplasmic and nuclear markers, respectively. (C) Endogenous mutp53 specifically interacts with the activated p-Ser326 form of HSF1 after heat shock. Nuclear fractions of MDA231 cells were precipitated with antibodies to HSF1, p-Ser326 HSF1 or GFP. Immunoblot loading normalized for similar amounts of immunoprecipitated total HSF1. (D) HSF1 does not interact with wildtype p53. Cell lysates from heat shocked HCT116 p53+/+ or MDA213 cells were immunoprecipitated for HSF1 or GFP and blotted for HSF1 and p53.


A
relative Hsp70 mRNA level
***
MDA231
MDA231 shp53
HS
B
MDA231
**
HSF1 reporter activity
sip53
HS
C
*
relative mRNA levels
H1299
H1299 175
Hsp70
HSF1
D
*
fold increase in relative HSF1 reporter activity
H1299 vector
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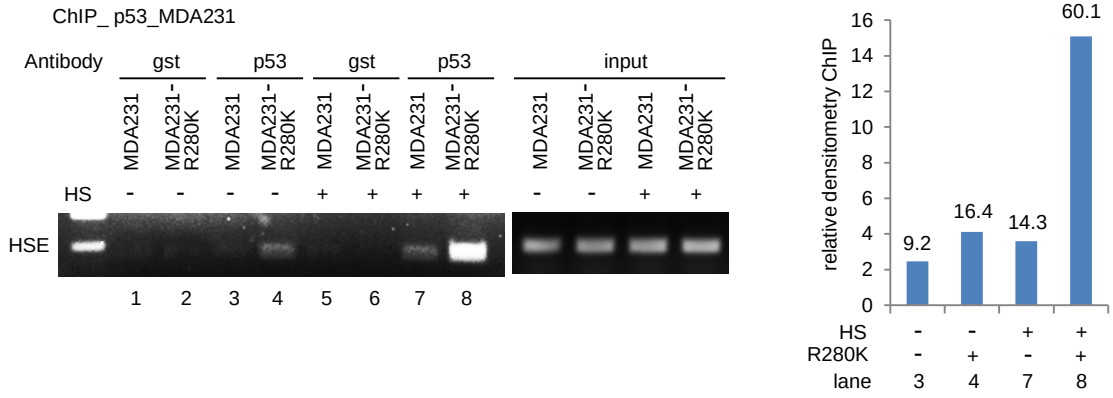


H

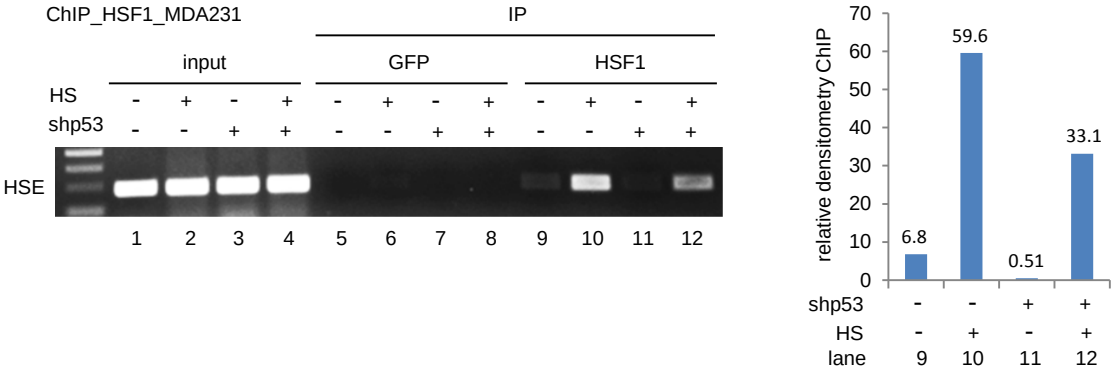


Figure 15. Mutant p53 protein enhances binding of HSF1 to HSE elements and stimulates its transcriptional activity (A) Tet-inducible mutp53 knockdown inhibits the heat shock response at the transcriptional level. Hsp70 mRNA levels measured by qRT-PCR +/- HS. (B) Mutp53 depletion inhibits HSF1 transcriptional activity upon heat shock. Scrambled (-) and p53 (+) siRNA were transfected with HSE-Luc and Renilla-Luc plasmids into MDA231 cells and analyzed 48 h later. (C-E) mutp53 expression stimulates HSF1 transcriptional activity. mutp53R175H stably expressed in H1299 cells after heat shock. Hsp70 and HSF1 mRNA levels via qRT-PCR (C) and fold increase in HSE-Luc reporter activity (D). HSE-Luc and Renilla-Luc plasmids transfected into MDA231 cells that stably express vector or native mutp53R280K -/+ HS. *p<0.05, **p<0.01, ***p<0.001, Student's t test. (F) Cross-linked chromatin derived from heat shocked mutp53 harboring MDA231 and MDA468 cells was subjected to ChIP analysis using the indicated antibodies. The occupancy of Hsp27, Hsp90 and Hsp70 promoters was analyzed by qPCR using specific HSPs primers. The myoglobulin promoter served as an internal negative control. Total qPCR product versus ChIP qPCR ratio is presented. (G, H) mutp53 protein promotes physical recruitment of HSF1 to its Heat Shock Element (HSE) in the Hsp70 target gene promoter. Chromatin immunoprecipitation assays from (G) MDA231 vector vs MDA231R280K cells with anti-p53 or irrelevant GST antibodies; and (H) from MDA231vector vs Tet-inducible MDA231-shp53 cells with anti-HSF1 or irrelevant GFP antibodies. Left A representative panel from 3 separate analyses each, with (right) densitometry of PCR products relative to input.

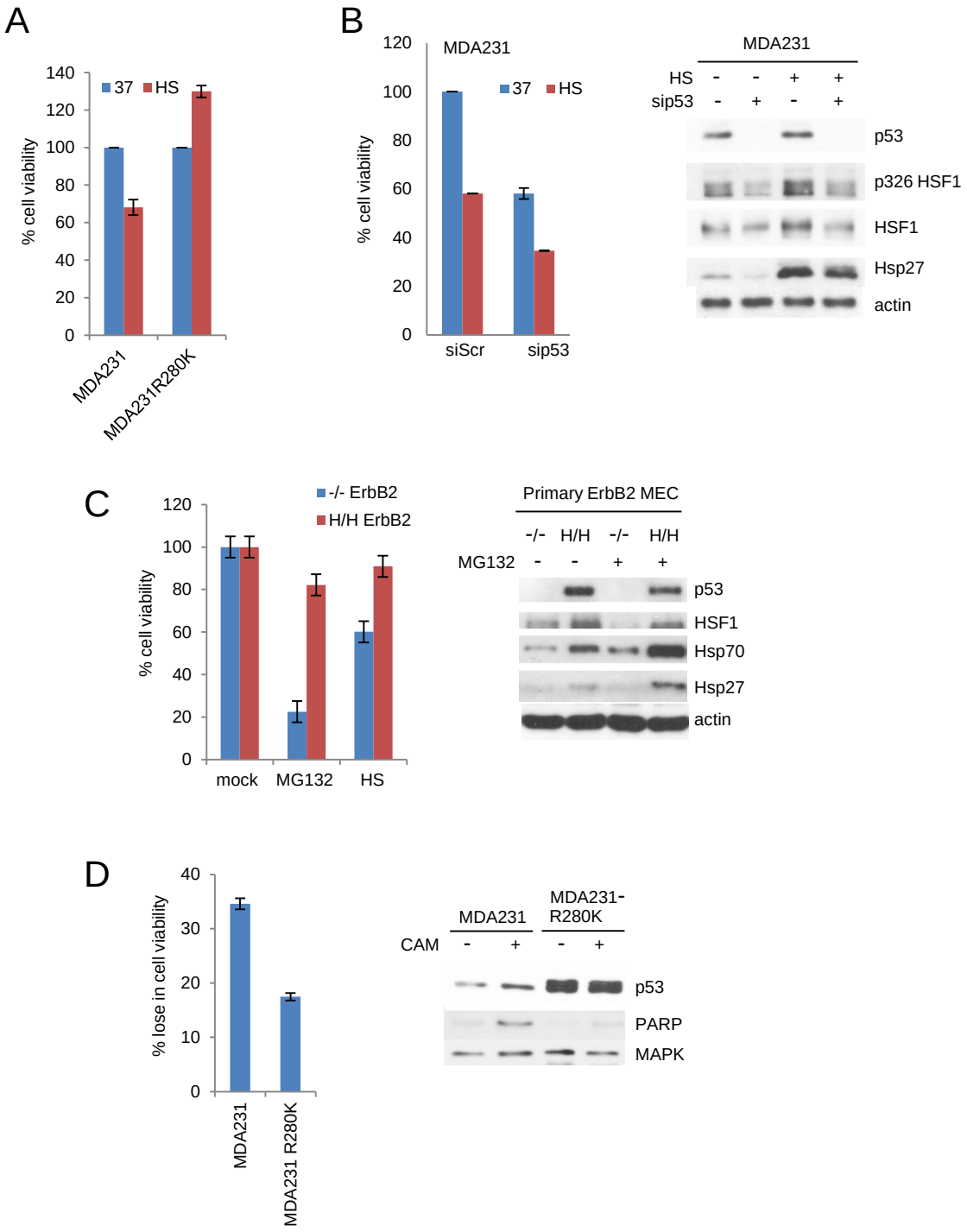


Figure 16. HSF1 activation by mutant p53 renders cells resistant to proteotoxic stress (A) The elevated levels of mutp53 in MDA231R280K cells bestow increased thermotolerance compared to parental controls. Cells were grown at 37°C or heat shocked for 1h at 43°C. After 48h viability was measured by CTB assay. (B) Conversely, downregulation of mutp53 decreases thermotolerance. Scrambled or p53 siRNA were transfected into MDA231 cells. After 24h post transfection cells were heat shocked for 1h at 43°C. Left, CTB assay after 48h. Right, corresponding immunoblot. (C) Primary mammary epithelial cells (MECs) from p53H/H;ErbB2 mice acquire higher tolerance to proteotoxic stress from heat shock (1h 43°C) or proteasome inhibition (MG132, 1uM for 24h) and exhibit higher levels of HSF1 and its targets Hsp70 and Hsp27, compared to p53-/-;ErbB2 MECs. (D) The elevated levels of mutp53 in MDA231R280K cells promote their chemoresistance to genotoxic Camptothecin (50 nM, 48h). Viability assay and PARP cleavage. MAPK, loading control. (A-D) Error bars represent mean +/- SD.

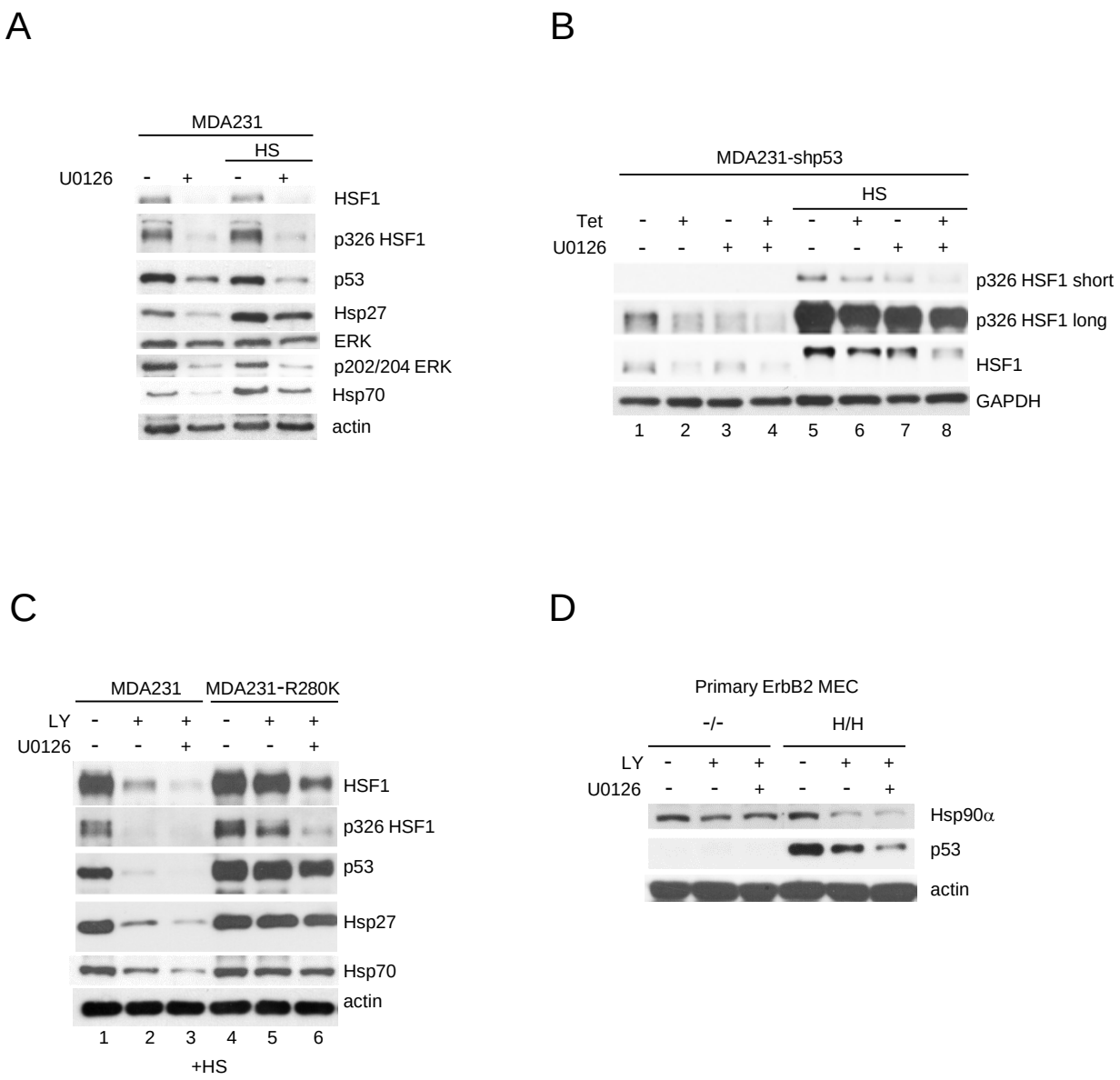
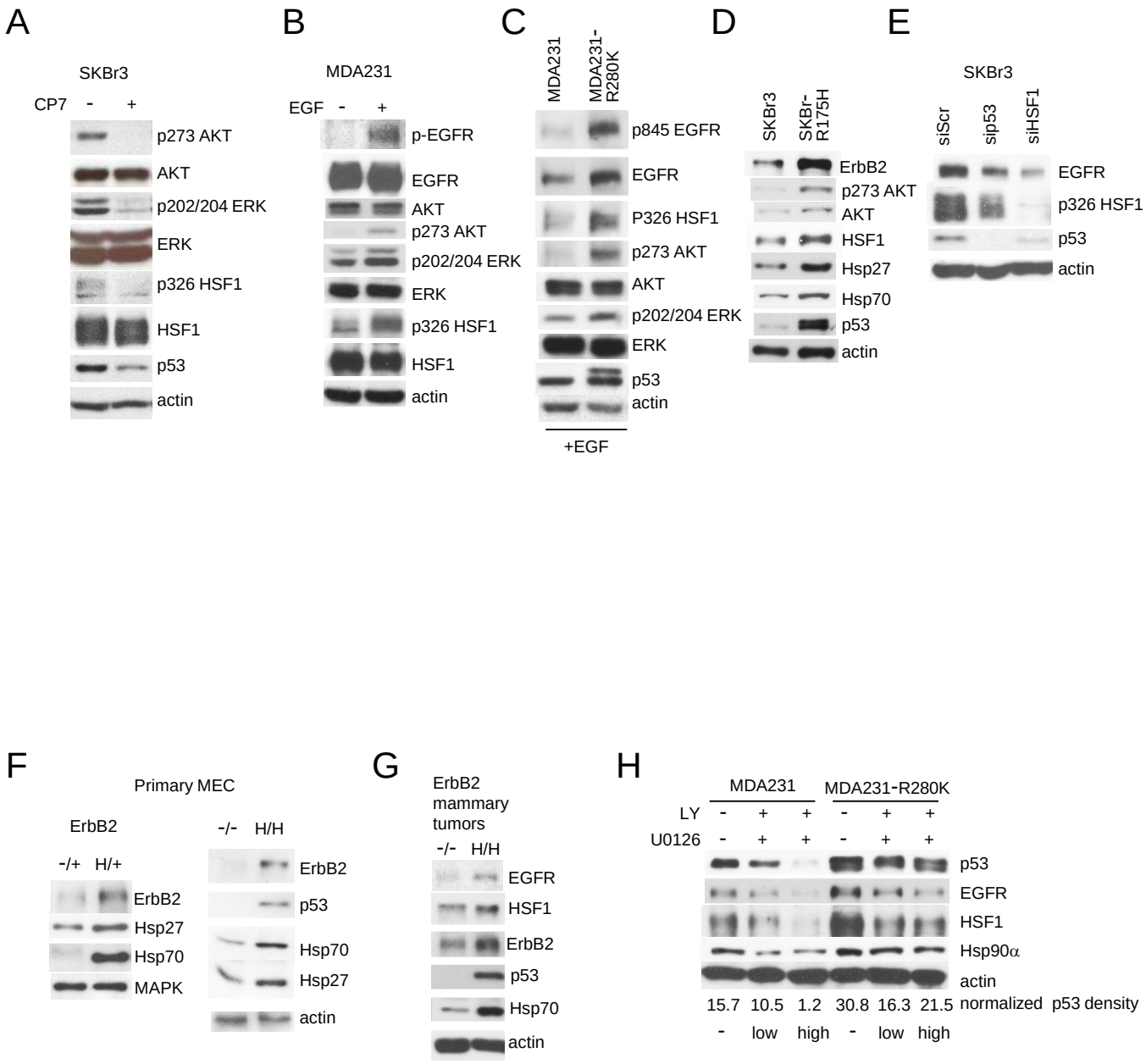


Figure 17. MAPK and PI3K cascades mediate HSF1 activation in a mutp53-dependent manner (A) MEK1/2 inhibition inhibits HSF1 activation in cancer cells. Immunoblot analysis of MDA231 cells treated overnight with 10μM U0126 or DMSO, followed by HS (43°C for 1h). (B) U0126 inhibition of HSF1 activation is more pronounced in mutp53-depleted cells. MDA231 stably expressing tet-inducible shp53 either vehicle (-) or tetracycline (+) treated, followed by 20μM U0126 for 24h and -/+ HS (43°C for 1h). (C) Dual inhibition of the PI3K and MAPK pathways further impedes HSF1 activation after heat shock. Combined treatment of MDA231 with U0126 (20uM) and LY294002 (5uM) for 24h. Inhibition is largely rescued by mutp53 overexpression in MDA231R280K cells. (D) As a result of HSF1 inhibition, PI3K (LY294002) and MAPK (UO126) inhibition reduces the levels of Hsp90alpha only in mutp53 MECs but not in p53 null MECs from ErbB2 mice (10uM for 24h each).


A
SKBr3
CP7 - +
p273 AKT
AKT
p202/204 ERK
ERK
p326 HSF1
HSF1
p53
actin
B
MDA231
EGF - +
p-EGFR
EGFR
AKT
p273 AKT
p202/204 ERK
ERK
p326 HSF1
HSF1
actin
C
MDA231
MDA231-R280K
p845 EGFR
EGFR
P326 HSF1
p273 AKT
AKT
p202/204 ERK
ERK
p53
actin
+EGF
D
SKBr3
SKBr-R175H
ErbB2
p273 AKT
AKT
HSF1
Hsp27
Hsp70
p53
actin
E
SKBr3
siScr
sip53
siHSF1
EGFR
p326 HSF1
p53
actin
F
Primary MEC
ErbB2
-/+ H/+
ErbB2
Hsp27
Hsp70
MAPK
-/- H/H
ErbB2
p53
Hsp70
Hsp27
actin
G
ErbB2 mammary tumors
-/- H/H
EGFR
HSF1
ErbB2
p53
Hsp70
actin
H
MDA231
MDA231-R280K
LY - + + - + +
U0126 - + + - + +
p53
EGFR
HSF1
Hsp90α
actin
15.7 10.5 1.2 30.8 16.3 21.5 normalized p53 density
- low high - low high

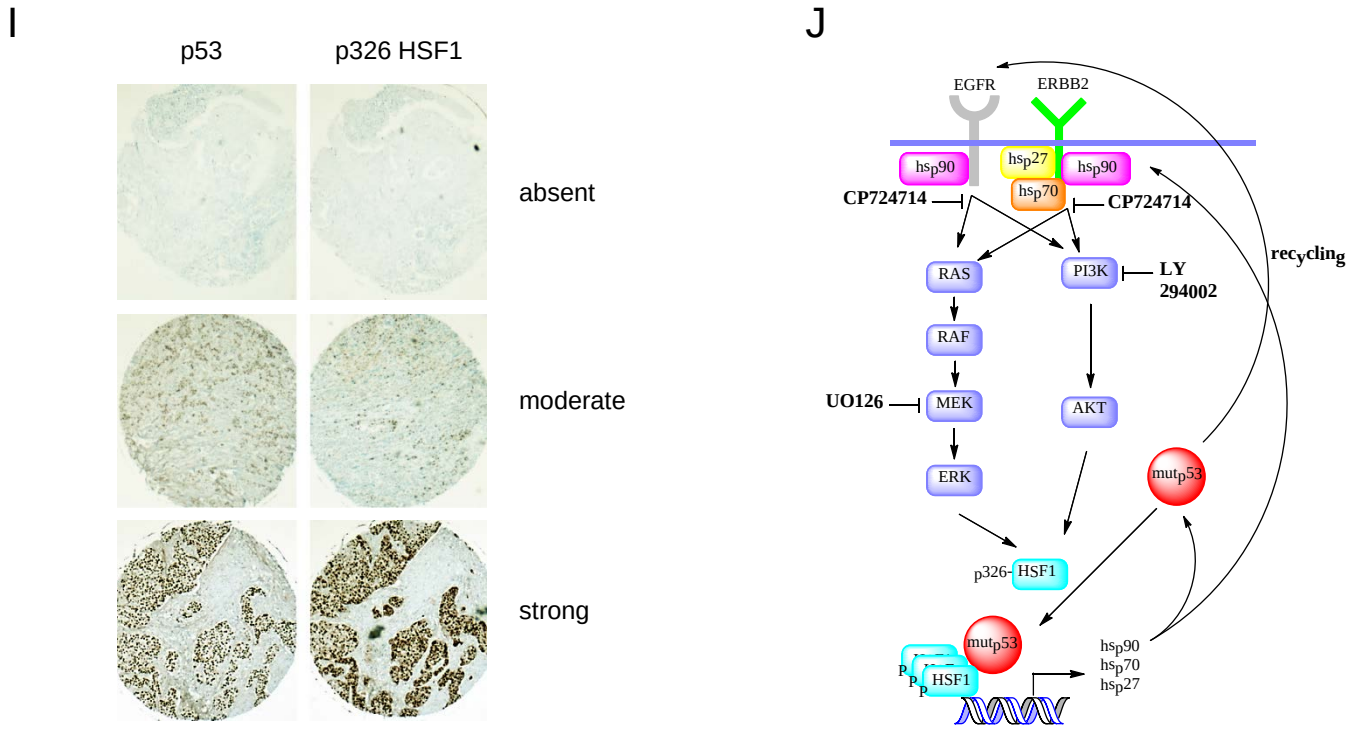


Figure 18. Upstream EGFR/ErbB2 signaling mediates HSF1 activation in a mutp53-dependent manner (A) Dual inhibition of EGFR/ErbB2 inhibits HSF1 activation. ErbB2+ breast cancer cells SKBr3 were treated with CP724714 (10 uM for 24h) or vehicle. (A-H) Immunoblot analysis. (B) Stimulation of EGFR signaling induces Ser326-HSF1 phosphorylation. MDA231 cells serum starved overnight and treated with EGF (30 ng/ml) for 10 min prior to harvesting. (C) Overexpression of mutp53 potentiates EGFR signaling after EGF stimulation. Increased phosphorylation of EGFR-Tyr845 and downstream effectors in MDA231R280K vs vector controls. Treatment as in (B). (D) Stable overexpression of native p53R175H in SKBr3 increases the levels of ErbB2, pAKT and the HSF1 response. (E) Depletion of mutp53 or HSF1 in SKBr3 cells reduces both EGFR and p-Ser326 HSF1 levels. Transfection with scrambled, p53 or HSF1 siRNAs. (F) Left, The mutp53 allele in MECs from p53H/+;ErbB2 mice correlates with increased levels of ErbB2 and higher activity of HSF1, compared to the p53 null allele. Right, even in the absence of the ErbB2 transgene, MECs from p53H/H mice have higher levels of ErbB2 and heat shock proteins than p53-/- MECs. (G) Primary mammary tumors from p53H/H;ErbB2 mice show higher levels of ErbB2 and EGFR than p53-/-;ErbB2 control tumors. (H) Dual inhibition of PI3K and MAPK signaling decreases EGFR levels in a mutp53-dependent manner. MDA231 vector and MDA231R280K cells were treated with low or high concentrations of UO126 (10 or 20μM) and LY 294002 (5 or 10μM) for 48h. (I) p53 and p-Ser326 HSF1 immunohistochemical staining of tissue microarray of 150 primary invasive human breast cancers from 75 different patients (2 biopsies from separate tumor regions per patient). Representative cases are shown. Strong p53 staining was used as surrogate marker for p53 mutation and a nuclear signal with a p-Ser326-specific HSF1 antibody as marker for HSF1 activation. Staining intensities were blindly scored from 0 to 4. A clear correlation between levels of mutp53 stabilization and nuclear p-Ser326 HSF1 were present in strong (3+) Her2-positive tumors (rho= 0.213, p = 0.008; n=85 cores), but not in Her2 weak or negative tumors (rho = 0.243, p=0.932; n= 65 cores). (J) Proposed model. mutp53, by enhancing EGFR/ErbB2/MAPK/PI3K signaling, potentiates HSF1 activity via a feed forward circuit and thereby endows cancer cells with superior tolerance to proteotoxic stress.