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A gain-of-function mutant p53–HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress

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To overcome proteotoxic stress inherent to malignant transformation, cancer cells induce a range of adaptive mechanisms, with the master transcription factor heat-shock factor 1 (HSF1)-orchestrated response taking center stage. Here we define a novel gain-of-function of mutant p53 (mutp53), whereby mutp53-overexpressing cancer cells acquire superior tolerance to proteotoxic stress. mutp53 via constitutive stimulation of EGFR and ErbB2 signaling hyperactivates the MAPK and PI3K cascades, which induce stabilization and phosphoactivation of HSF1 on Ser326. 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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TP53 is the most frequently mutated gene in human cancer, with over 50% of all tumors carrying p53 mutations in the DNA-binding domain.[1] Aside from acquiring dominant-negative activity over the remaining wild-type (wt) allele,[2] mutant p53 (mutp53) proteins not only lose their tumor suppressor activities but also gain new oncogenic functions (gain-of-function) that actively contribute to malignant progression by promoting tumor growth, metastasis and chemoresistance.[1–4] Guarding the proteome against misfolding and aggregation induced by proteotoxic stress due to heat, ROS, hypoxia, acidosis, DNA damage and aneuploidy, several stress-induced heat-shock proteins (HSPs) including Hsp90, Hsp70 and Hsp27 are essential in guiding proper refolding of stress-misfolded 'client' polypeptides. The transcription of inducible HSPs is regulated by the master transcription factor heat-shock factor 1 (HSF 1).[5,6] 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 environment. 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.[7,8] Importantly, recent studies on HSF1 knockout mouse model 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.[8] However, biochemical basis of this cooperation 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.

Here we identify a novel gain-of-function of mutp53 as a promoter of HSF1 activity. 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 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.

Results

Mutp53 upregulates the HSF1 protein and augments the heat-shock response. We previously showed that HSF1 controls the stability of mutp53 protein in human cancer cells

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Abbreviations: HS, heat shock; HSP, heat-shock protein; mutp53, mutant p53; wtp53, wild-type p53; HSF1, Heat-Shock Factor 1; HSE, heat-shock elements; GOF, gain-of-function; EGFR, epidermal growth factor receptor; MEC, mammary epithelial cells; ChIP, chromatin immunoprecipitation; Luc, luciferase; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase
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via activation of Hsp90, which strongly stabilizes mutp53 (Li *et al*.[9,10] and Figure 1a). Conversely, here we 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 (Figure 1a). 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 (Figure 1b). Conversely, excess ectopic native mutp53 R280K in MDA231 cells further increases the HSF1 targets Hsp70/Hsp27 upon HS (Figure 1c). Likewise, expression of mutp53R175H in p53 null H1299 cells induces HSF1 and Hsp70/Hsp27 (Figure 1d), further enhanced by HS at the protein (Figure 1d) and mRNA (Figure 4c) levels. Upregulation of HSF1 by mutp53 appears to be generic, as it is observed in response to different p53 mutants (Figures 1d and e). To confirm these results in mice we generated a novel breast cancer model by introducing the well-characterized p53 R172H allele (H thereafter)[11,12] 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; Figure 1f).

Thus, these data indicate that mutp53 positively regulates HSF1 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 (Figure 4c), 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

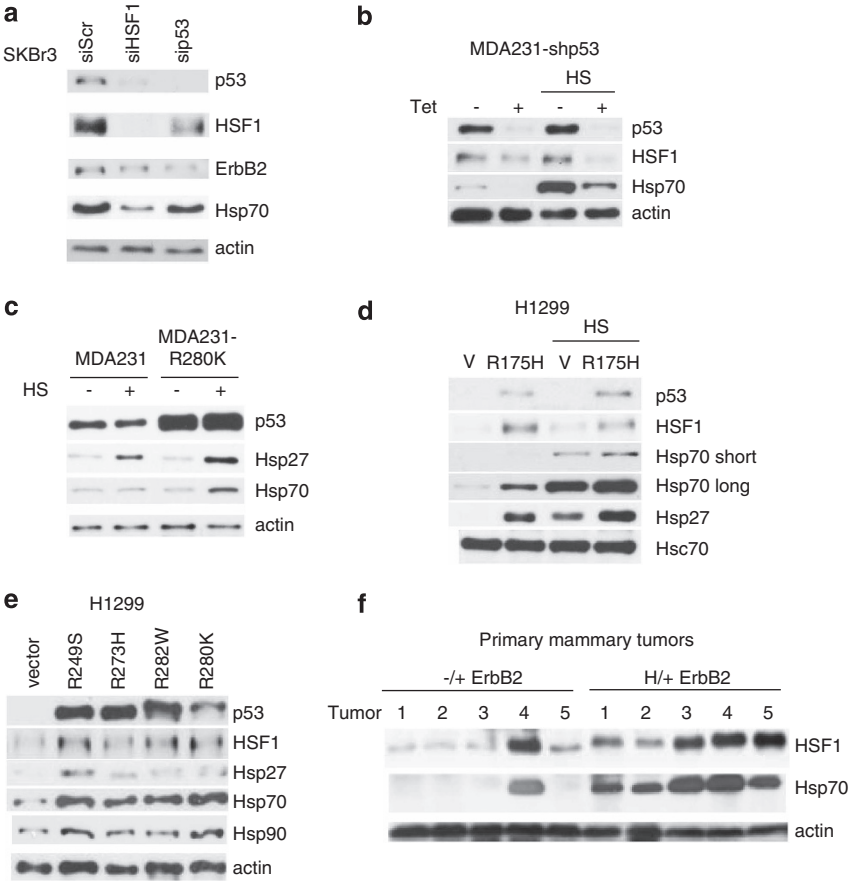


Figure 1 Mutant p53 upregulates HSF1 protein and augments the heat-shock response. (**a**) siRNA-mediated depletion of mutp53 in SKBr3 cells results in downregulation of HSF1 and its transcriptional target Hsp70. siHSF1 and scrambled siRNA as controls. (**a**–**c**, **e** and **f**) actin, loading control. (**b**) Tet-inducible p53 shRNA downregulates HSF1 and its target Hsp70 and is further enhanced by HS (43 °C, 1 h). Stable MDA231-shp53 cells. (**c**) Conversely, excess ectopic native mutp53 R280K in MDA231 cells further increases the HSF1 targets Hsp70/Hsp27 upon HS. (**d**) Ectopic mutp53R175H in p53 null H1299 cells upregulates HSF1 and its targets Hsp70/Hsp27, which is further enhanced by HS. Hsc70, loading control. (**e**) Upregulation of HSF1 and its targets in response to all tested p53 mutants. (**f**) Compared with littermate p53 $-/+$;ErbB2, primary mammary tumors from p53H/$+$;ErbB2 mice show increased levels of HSF1 and Hsp70. Actin as a loading control

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.[14] Furthermore, Ser326 phosphorylation protects HSF1 from polyubiquitination and proteosomal degradation, causing HSF1 to stabilize.[15] 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 (Figure 2a). 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 (Figure 2b). HS induced nuclear translocation and p-Ser326-phosphorylation of HSF1, which was augmented in MDA231R280K compared with control cells (Figure 2b). Conversely, shRNA-mediated downregulation of mutp53 decreased (mainly cytoplasmic) total HSF1 of unstressed cells and decreased HS-activated nuclear p-Ser326 HSF1 (Figure 2c). 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 (Figure 2d).

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.[4] 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 (Figure 3a). Importantly, in reciprocal co-immunoprecipitations from MDA231 cells an endogenous nuclear mutp53-HSF1

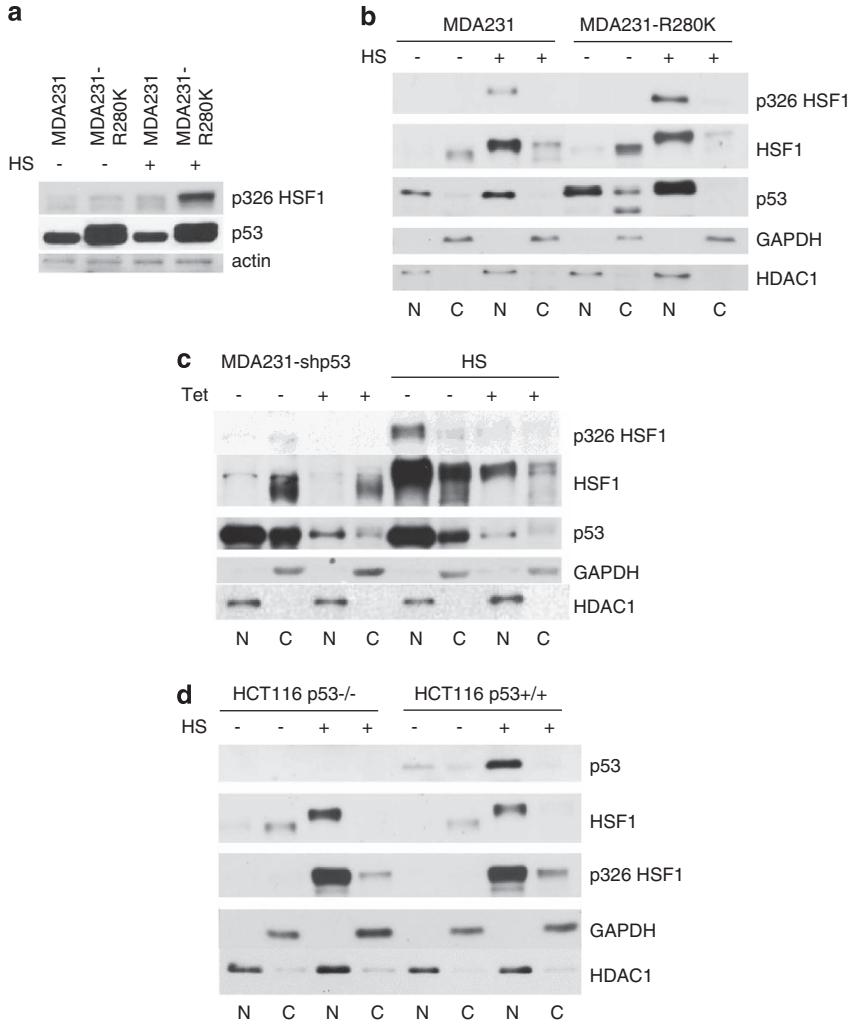


Figure 2 Mutant p53 promotes HSF1 activation via Ser326 phosphorylation. (**a**) Activated p-Ser326 HSF1 is greatly upregulated in MDA231R280K cells after HS (43 °C, 1 h). Actin is the loading control. (**b**) MDA231R280K cells induce higher levels of activated nuclear p-Ser326 HSF1 after HS than vector controls. Note that the slower migration of HS-activated HSF1 detectable in 6% SDS PAAG 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 HS (43 °C, 1 h) compared with vehicle-treated control cells. Note that the slower migration of HS-activated HSF1 detectable in 6% SDS PAAG is due to phosphorylation. (**d**) HSF1 is specifically upregulated by mutant but not wild-type p53. No increase in HSF1 levels or activation was seen in HCT116 p53 $-/-$ *versus* p53 $+/+$ cells. The slower migration of total HSF1 detectable in 6% SDS PAAG upon HS is due to phosphorylation. (**b–d**) N – nuclear fraction, C – cytoplasmic fraction. GAPDH and HDAC1 as cytoplasmic and nuclear markers, respectively

complex, enhanced by HS, was confirmed (Figure 3b). 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 (Figure 3c). We failed to detect the wtp53-HSF1 complex in HCT116 p53+/+ cells despite HS, indicating that HSF1 interaction is a mutp53-specific trait (Figure 3d).

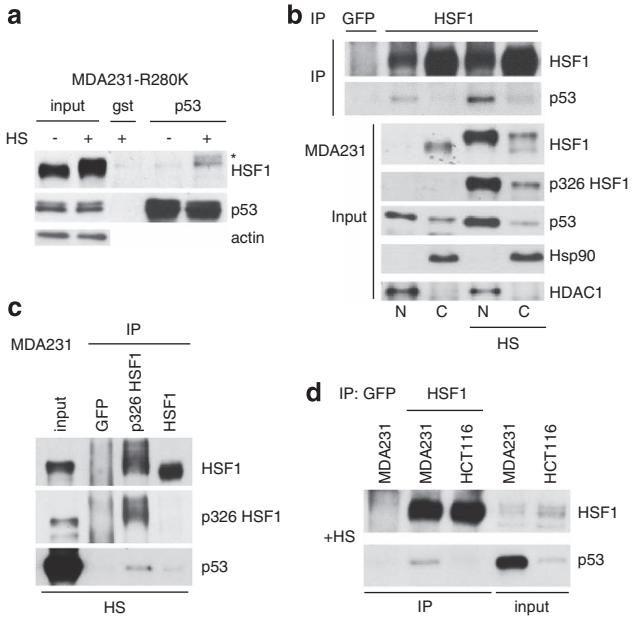


Figure 3 Mutant p53 interacts with activated HSF1 in the nucleus. (**a**) mutp53-HSF1 complexes are induced by HS (43 °C, 1 h). Whole-cell lysates of MDA231R280K cells were immunoprecipitated with p53 or irrelevant GST antibodies and immunoblotted for HSF1 and p53. * – the slower migration band of total HSF1 detectable in 6% SDS PAAG corresponds to phosphorylated form of HSF1. (**b**) Likewise, the endogenous mutp53-HSF1 complex in the nucleus is induced by HS. 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 HS. 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 wild-type p53. Cell lysates from heat-shocked HCT116 p53+/+ or MDA213 cells were immunoprecipitated for HSF1 or GFP and blotted for HSF1 and p53

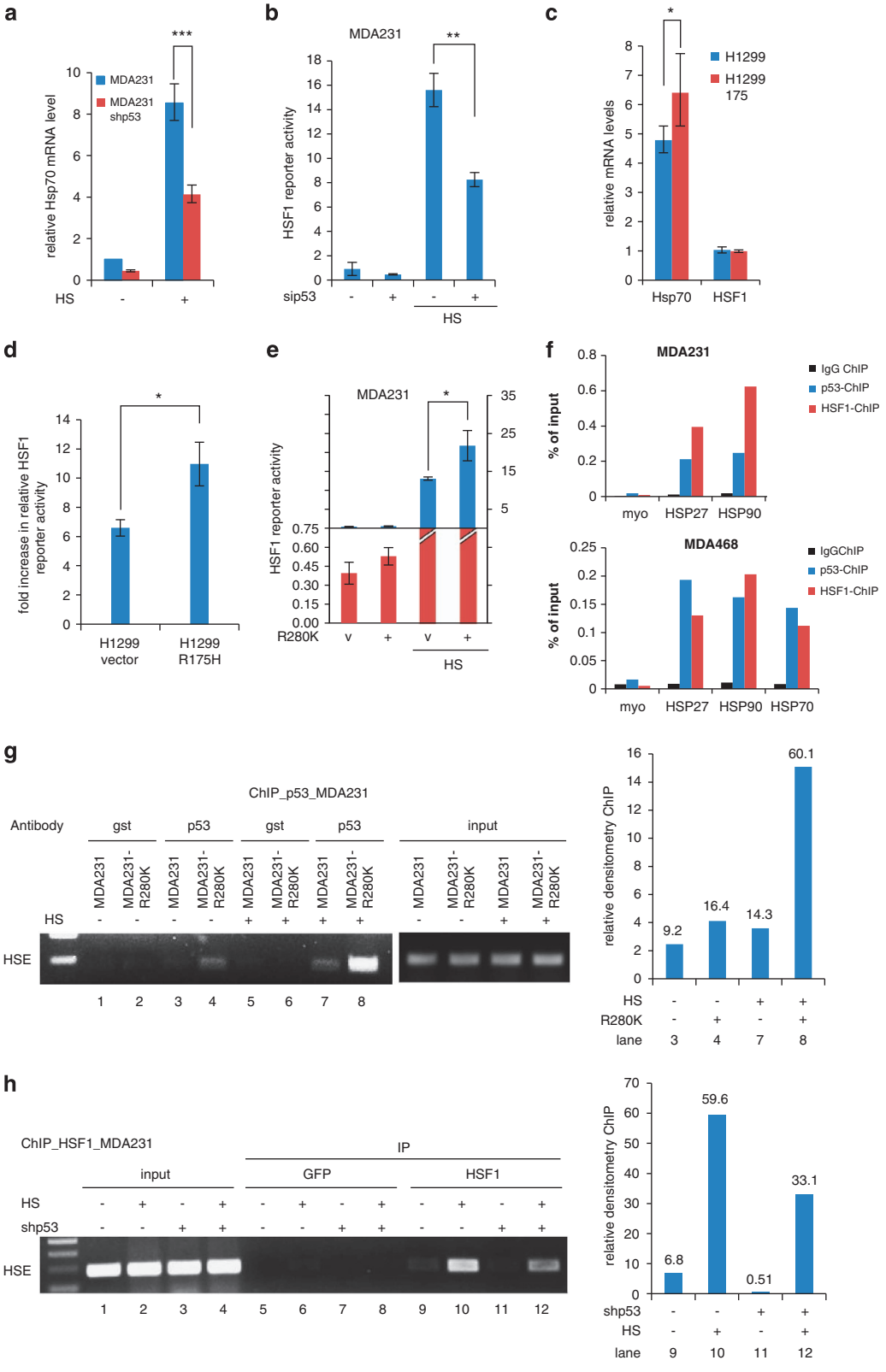
Mutp53 protein enhances binding of HSF1 to HSE elements and stimulates its transcriptional activity. The fact that mutp53 preferably interacts with activated HSF1 (Figures 3b and c) suggests that it may modulate the transcriptional activity of HSF1. Therefore, we examined the mRNA levels of Hsp70 after shRNA-mediated mutp53 knockdown. As expected, HS-mediated proteotoxic stress strongly induced Hsp70 mRNA (Figure 4a) and protein (Figures 1b–d) in MDA231 cells. Importantly, in mutp53-depleted cells mRNA induction was suppressed by 50%, especially upon HS with maximum HSF1 activity (Figure 4a). These results were confirmed by HSF1 reporter assays using HSE-luciferase (Figure 4b). Conversely, in H1299 cells expressing mutp53R175H Hsp70, mRNA (Figure 4c) and protein (Figure 1d) were induced compared with vector controls, whereas the HSF1 transcript level itself was unresponsive to mutp53 (Figure 4c). In support, HSE-Luc reporter activation was stimulated by 40% in H1299 cells expressing mutp53R175H compared with controls (Figure 4d). Likewise, the elevated levels of mutp53 in MDA231R280K cells increased HSE-Luc activity upon HS (Figure 4e). Thus, mutp53 is an important enhancer of the HSF1 transcriptional activity.

Next, we asked whether mutp53 proteins directly contribute to HSF1's physical recruitment to HSF1-binding sites. Chromatin immunoprecipitation (ChIP) analyses in heat-shocked MDA231 and MDA468 cells showed that mutp53 is bound with similar efficiency to Hsp27, Hsp90 and Hsp70 promoters as HSF1 (Figure 4f). Moreover, HSF1 binding to HSE is mutp53-dependent, as HSF1 recruitment to HSE was greatly enhanced in the MDA231R280K compared with MDA231 control cells (Figure 4g, lanes 7, 8). On contrary, HSF1 binding to HSE was reduced in mutp53-depleted cells before (Figure 4h, lanes 9, 11) and after HS (Figure 4h, 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, we 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

Figure 4 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. Two independent experiments were performed in triplicate. $*P<0.05$, $**P<0.01$, $***P<0.001$, Student's *t*-test. (**b**) Mutp53 depletion inhibits HSF1 transcriptional activity upon HS. Scrambled (−) and p53 (+) siRNA were transfected with HSE-Luc and Renilla-Luc plasmids into MDA231 cells and analyzed 48 h later. Two independent experiments were performed in triplicate. (**c**–**e**) mutp53 expression stimulates HSF1 transcriptional activity. mutp53R175H transiently expressed in H1299 cells. qRT-PCR of Hsp70 and HSF1 after HS (**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. Two independent experiments were performed in triplicate. $*P<0.05$, 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 with qPCR using specific HSPs primers. The myoglobulin promoter served as an internal negative control. Total qPCR product *versus* ChIP qPCR ratio is presented. (**g** and **h**) mutp53 protein promotes physical recruitment of HSF1 to its 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 MDA231 vector *vs* Tet-inducible MDA231-shp53 cells with anti-HSF1 or irrelevant GFP antibodies. *Left* A representative panel from three separate analyses each, with (*right*) densitometry of PCR products relative to input

cells exhibited higher levels of activated HSF1 (Figures 2a and b), with subsequent upregulation of Hsp70/Hsp27 (Figure 1c). Importantly, MDA231R280K acquired higher thermotolerance, indicated by higher cell viability, compared with MDA231 control cells (Figure 5a). Whereas downregulation of mutp53 by siRNA decreases levels of activated HSF1 and its



transcriptional target Hsp27 and confers increased sensitivity to HS (Figure 5b). 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 (Figure 5c). 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.[7] Thus, we 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 (Figure 5d).

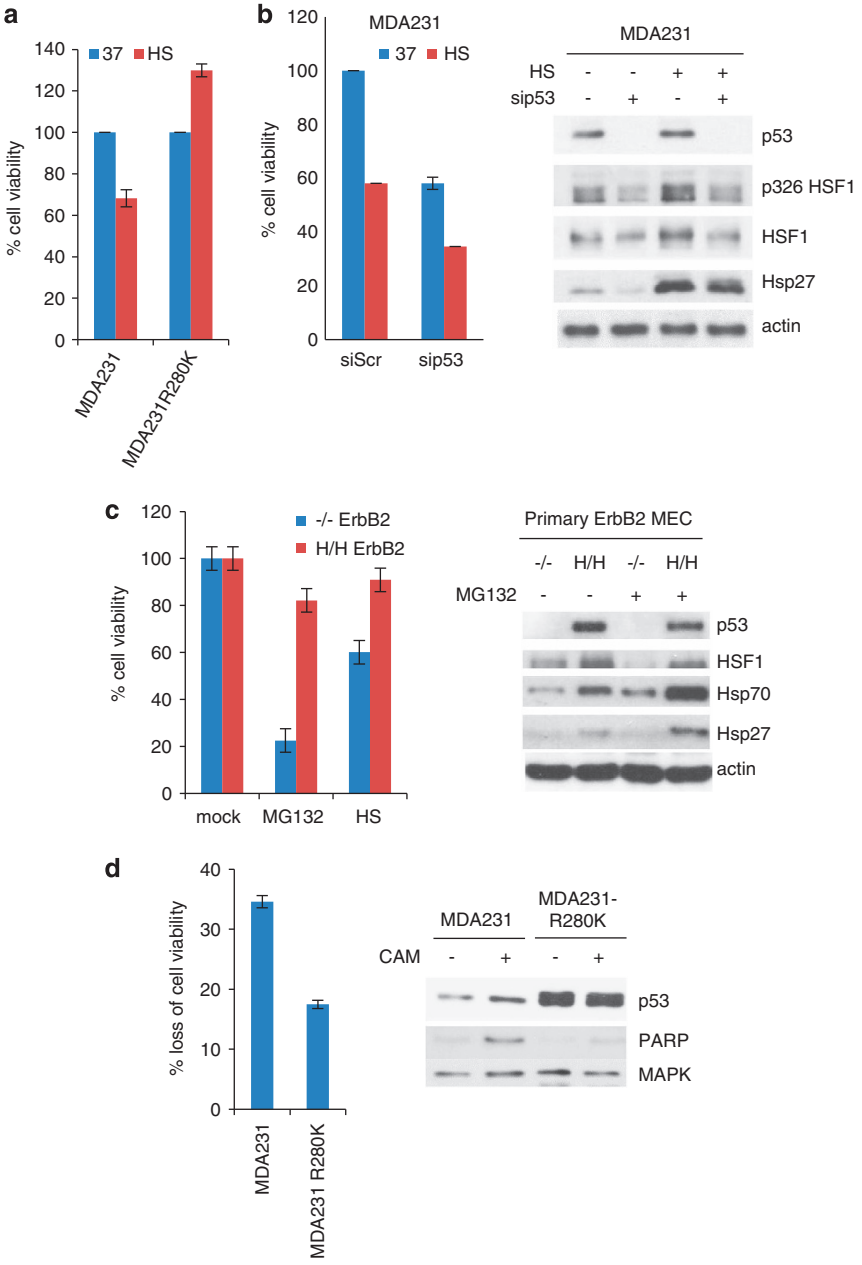


Figure 5 HSF1 activation by mutant p53 renders cells resistant to proteotoxic stress. (**a**) The elevated levels of mutp53 in MDA231R280K cells bestow increased thermotolerance compared with parental controls. Cells were grown at 37 °C or heat-shocked for 1 h at 43 °C. After 48 h, viability was measured using CTB assay. Two independent experiments were performed in triplicate. (**b**) Conversely, downregulation of mutp53 decreases thermotolerance. Scrambled or p53 siRNA was transfected into MDA231 cells. After 24-h post transfection, cells were heat-shocked for 1 h at 43 °C. *Left,* CTB assay after 48 h. *Right,* corresponding immunoblot. (**c**) Primary MECs from p53H/H;ErbB2 mice acquire higher tolerance to proteotoxic stress from HS (1 h, 43 °C) or proteasome inhibition (MG132, 1 μM for 24 h) and exhibit higher levels of HSF1 and its targets Hsp70 and Hsp27, compared with p53 − / − ;ErbB2 MECs. Two independent experiments were performed in triplicate. (**d**) The elevated levels of mutp53 in MDA231R280K cells promote their chemoresistance to genotoxic Camptothecin (50 nM, 48 h). Viability assay and PARP cleavage. MAPK, loading control. (**a**–**d**) Error bars represent mean ± S.D.

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. We identified the selective MEK 1/2 inhibitor UO126[16] and the PI3K inhibitor LY294002[17] as potent suppressors of HSF1 Ser326 phosphorylation (Figure 6). The levels of total and active HSF1 dramatically decreased in MDA231 cells after UO126 treatment in the absence and presence of HS, causing a parallel decline in Hsp70 and Hsp27 (Figure 6a). Notably, mutp53 depletion sensitizes cells for UO126's ability to suppress HSF1 activation (Figure 6b, 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 (Figure 6c). Moreover, dual inhibition of PI3K and MAPK cascades by combined drug treatment further impeded HSF1 levels and expression of its target genes upon HS (Figure 6c, lanes 2, 3). Conversely, the inhibition of the HSF1 response imparted by these drugs was largely rescued by overexpression of mutp53 (Figure 6c), 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 (Figures 6a, c and 7h). Mutp53 levels also decreased upon LY294002/UO126 treatment in primary mutp53 H/H;ErbB2 MECs (Figure 6d). This effect is likely due to decreased transcriptional activity of HSF1, as our previous studies showed that HSF1 ablation destabilizes mutp53 via Hsp90 inhibition[9] (Figure 1a). Indeed, as a result of HSF1 inhibition, LY294002/UO126 treatment significantly reduced the levels of Hsp90α 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 (Figure 6d).

Hence, our 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 (Figure 6), we hypothesized that HSF1 Ser326-phosphorylation could be regulated by upstream EGFR and/or ErbB2 signaling in a mutp53-dependent manner. To test this notion, we treated SKBr3 cells with dual EGFR/ErbB2 tyrosine kinase inhibitor CP724714.[18] As expected, CP724714 inhibited phosphorylation of ERK/pERK and AKT/pAKT (Figure 7a). Similar to the effect of the PI3K/MAPK blockade (Figure 6), EGFR/ErbB2 inhibition by CP724714 also reduced levels of activated p-Ser326

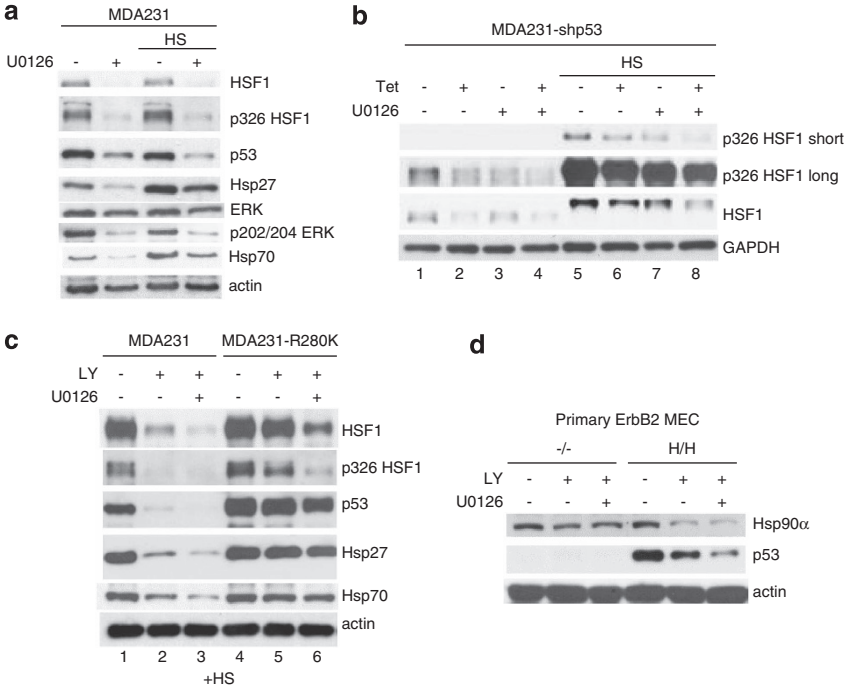
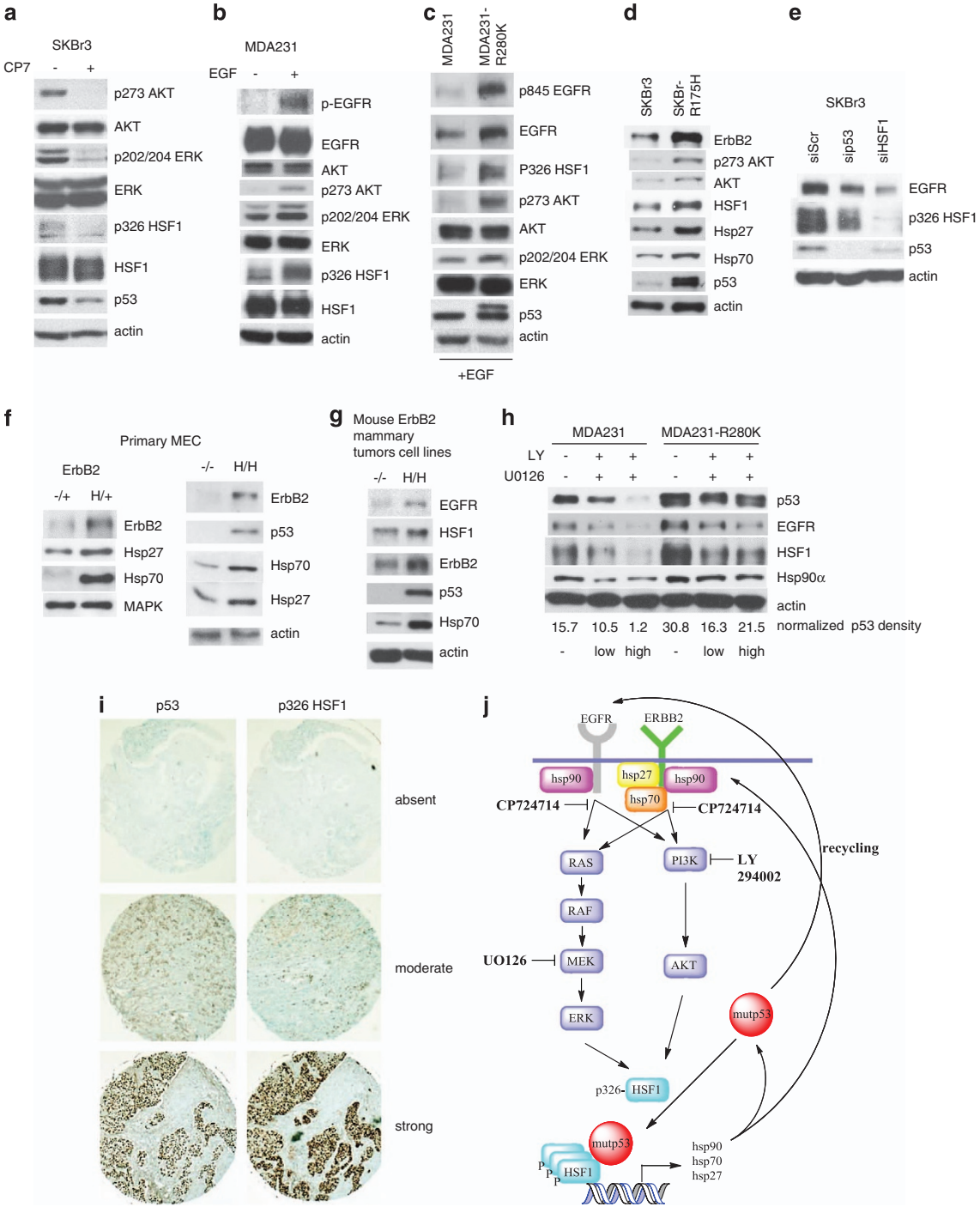


Figure 6 MAPK and PI3K cascades mediate HSF1 activation in a mutp53-dependent manner. (**a**) MEK 1/2 inhibition inhibits HSF1 activation in cancer cells. Immunoblot analysis of MDA231 cells treated overnight with 10 μM U0126 or DMSO, followed by HS (43 °C for 1 h). (**b**) UO126 inhibition of HSF1 activation is more pronounced in mutp53-depleted cells. MDA231 stably expressing tet-inducible shp53 either vehicle- ($-$) or tetracycline ($+$)-treated, followed by 20 μM U0126 for 24 h and $\pm$ HS (43 °C for 1 h). (**c**) Dual inhibition of the PI3K and MAPK pathways further impedes HSF1 activation after HS. Combined treatment of MDA231 with UO126 (20 μM) and LY294002 (5 μM) for 24 h. Inhibition is largely rescued by mutp53 overexpression in MDA231R280K cells. (**d**) As a result of HSF1 inhibition, PI3K (LY294002) and MAPK (UO126) inhibition significantly reduces the levels of Hsp90α only in mutp53 MECs but only marginally in p53 null MECs from ErbB2 mice (10 μM for 24 h each)

HSF1, leading to a decline in mutp53 levels (Figure 7a). 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 phosphorylation[19] not only induces AKT and ERK phosphorylation but also enhances Ser326 HSF1 phosphorylation (Figure 7b), 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, we 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 (Figure 7c). This effect was concomitant with induction of p-Ser326 HSF1 even in the absence of proteotoxic stress (Figure 7c), 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 (Figure 7d). Consistently, siRNA-mediated depletion of mutp53 in SKBr3 cells reduced both ErbB2 (Figure 1a) and EGFR levels and was accompanied by decreased p-Ser326 HSF1 (Figure 7e). Similar to p53 depletion, HSF1 knockdown also reduced the level of ErbB2 and EGFR (Figures 1a and 7e), consistent with ErbB2 and EGFR being well-established HSPs clients.[20,21] 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 7f, 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 (Figure 7f, 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 (Figure 7g). Importantly, dual inhibition of PI3K and MAPK signaling not only affected HSF1 activation (Figures 6c and 7a) but also decreased EGFR levels in MDA231 cells (Figure 7h). The fact that this dual inhibition was significantly less pronounced in mutp53-overexpressing MDA231R280K cells (Figure 7h) implies that mutp53 positively regulates the tyrosine kinase receptors ErbB2 and EGFR,[22] and via their downstream signaling affects HSF1 activation.

Together, our 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 our model, we found a clear correlation between p53 and nuclear p-Ser326 HSF1 staining only in strongly (3+) 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, $P = 0.932$; Figure 7i, Supplementary Figure 1).

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 (Figure 7j).

Discussion

Inherent to malignant transformation is massive perennial proteotoxic stress due to aneuploidy, ROS, hypoxia and acidosis.[6,23] To overcome proteotoxic stress, cancer cells mount a wide range of adaptive mechanisms in which the HSF1-orchestrated response has *the* central role. Aside from maintaining cellular homeostasis by stress-mediated induction of HSPs,[5] 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.[7] Not surprisingly, HSF1 is upregulated in 80% of breast cancers and is associated with high histologic grade and increased mortality.[24] 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%), and correlate with high rates of metastatic recurrence, chemoresistance and poor overall survival.[25] A critical cancer specific phenotype of mutp53 is increased its protein stability causing mutp53 accumulation in tumors, but not in normal tissues.[11] We 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.[9,26,27] Due to its high translational impact, the question of what causes tumor-specific mutp53 stabilization has recently attracted a lot of attention.[1] Previously we showed 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.[9] Thus, upregulation of HSF1 in cancer cells can mechanistically underlie the cancer-cell-specific stabilization of mutp53.

Conversely, here we 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

Figure 7 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 μM for 24 h) 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 before 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 with 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**) Cell lines, established from primary mammary tumors of H/H;ErbB2 mouse showed elevated levels of ErbB2, EGFR and activated HSF1 (indicated by increased Hsp70) compared with p53−/−;ErbB2 littermate control. (**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 20 μM) and LY294002 (5 or 10 μM) for 48 h. (**i**) p53 and p-Ser326 HSF1 immunohistochemical staining of tissue microarray of 150 primary invasive human breast cancers from 75 different patients (two 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). See also Supplementary Figure 1. (**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

p-Ser326 HSF1 to recruit HSF1 to its specific DNA-binding sites in target gene promoters and enhances its transcriptional program (Figure 4). 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 (Figure 5d), which inadvertently may stimulate adaptation to adverse environments and promote cancer cell survival and chemoresistance. Thus, the notorious chemoresistance of mutp53 cancer cells[28] can at least in part be attributed to the positive mutp53-HSF1 circuit (Figure 5d). 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.[29]

How exactly does mutp53 stimulate HSF1 activation? Recently, Dai *et al.*[15] demonstrated that HSF1 activation by Ser326 phosporylation depends on deregulated MAPK signaling. On the other hand, using ectopically expressed mutp53 in H1299 cells, Muller *et al.*[22] established that mutp53 drives enhanced EGFR recycling to the cell surface of cancer cells. 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.[30] Indeed, we identified mutp53-driven deregulated MAPK/ERK and PI3K signaling to be important effectors of HSF1 activation (Figures 6c and d), whereas inhibition of the upstream EGFR and ErbB2 receptors prevented HSF1 activation (Figure 7a). 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 (Figures 7c, g and e) and ErbB2 (Figures 1a and 7d). Conversely, HSF1 knockdown destabilizes both EGFR and ErbB2 (Figures 1a and 7d), consistent with the facts that EGFR is an established Hsp90 client[31] and that ErbB2 is stabilized by Hsp90, Hsp70/Hsc70 and Hsp27.[5,32] In aggregate, our results strongly imply that the EGFR and ErbB2 signaling, at least in part, is mediated by mutp53 in an HSF1-dependent manner. In support, we found a correlation between mutp53 and nuclear p-HSF1 levels only in strongly (3+) HER2-positive, but not in HER2-negative, ER/PR-positive human breast cancers (Figure 7i). Together, our 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, we delineate a novel gain-of-function of mutp53 defined by a mechanistic feed forward link between HSF1 and mutp53. We propose that mutp53, through enhanced recycling[22] 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 (Figure 7j). Hence, mutp53 initiates a feed forward loop that endows cancer cells more resistant to proteotoxic stress, providing a distinct survival advantage.

Materials and Methods

Cells. Human mutp53 breast cancer cells MDA231 (p53R280K), MDA468 (p53R273K), T47D (p53L194F), SKBr3 (p53R175H), colon cancer cells SW480 (p53 R273H/P309S) and isogenic HCT116 p53 −/− *vs* wtp53 +/+ cells were used. Generation of stable Tet-On shp53 MDA231 was described.[9] MDA231 and SKBr3 stably overexpressing native ectopic p53 (R280K and R175H) or vector only were generated by transfection followed by selection in G418. All cells were cultured in 10% FCS/DMEM and where indicated treated with CP724714 (Selleckchem, Huston, TX, USA), UO126, LY294002 (LC Labs, Woburn, MA, USA) and Camptothecin (Sigma, St. Louis, MO, USA). Viability was determined using CellTiter-Blue Assay, and HSF1 activity using Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) with the Hsp70 HSE-Luc reporter (Qiagen, Valencia, CA, USA).

RNA interference. Pools of four different siRNA duplexes specific for p53 or HSF1, or scrambled controls, were transfected with Lipofectamine (RNAiTM/RNAiMAX, Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 h later and analyzed.

ChIP assays. ChIP assays were performed as described in Denissov *et al.*[33] 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: 5′-AGTTTCTGA GAGCCCAGACC-3′, 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′-CCCTGGGCTTTTA-TAAGTCG-3′.[34]

Immunoblots and immunoprecipitations. For immunoblots, equal total protein of cell lysates (2.5–20 μg) were detected with antibodies to mouse p53 (FL393), human p53 (PAb1801; Santa Cruz Biotechnology), HSF1, p-Ser326 HSF1, AKT, pAKT, Erk, pErk, Hsp70, Hsp90, Hsp90α, EGFR, EGRF-Tyr845P (all Cell Signaling, Danvers, MA, USA), ErbB2, actin, GAPDH, GTS, GFP, HSc70 and HDAC1 (all Neomarkers, Fremont, CA, USA).[35–37] SDS-PAAG gels (6%) were used to detect slower migrating HS-activated HSF1 used in experiments presented in Figures 2 and 3. SDS PAAG (10%) gels were used in all other experiments. Co-immunoprecipitations were performed with 1 μg of antibody overnight. Beads were washed with SNNTE and RIPA buffers 3 × each, followed by blotting.

Cell fractionation. Cells were harvested, rinsed, pelleted, resuspended in 5 vol of cold CARSB buffer (10 mM Tris pH 7.5, 1.5 mM $CaCl_2$, 10 mM NaCl, protease inhibitor cocktail, 10 mM 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 1000 × *g*. The supernatant (cytoplasmic fraction) was adjusted to 200 mM NaCl. The crude nuclear pellet was suspended in buffer C (10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 400 mM NaCl, 0.5% Triton X-100, protease inhibitor cocktail, 10 mM sodium orthovanadate) and sonicated. The homogenate was centrifuged for 15 min at 16 000 × *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 °C 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-μm 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 or strong.

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

Conflict of Interest

The authors declare no conflict of interest.

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