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Mutant p53 Reprograms TNF Signaling in Cancer Cells through Interaction with the Tumor Suppressor DAB2IP

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SUMMARY

Inflammation is a significant factor in cancer development, and a molecular understanding of the parameters dictating the impact of inflammation on cancers could significantly improve treatment. The tumor suppressor p53 is frequently mutated in cancer, and p53 missense mutants (mutp53) can acquire oncogenic properties. We report that cancer cells with mutp53 respond to inflammatory cytokines increasing their invasive behavior. Notably, this action is coupled to expression of chemokines that can expose the tumor to host immunity, potentially affecting response to therapy. Mechanistically, mutp53 fuels NF-κB activation while it dampens activation of ASK1/JNK by TNFα, and this action depends on mutp53 binding and inhibiting the tumor suppressor DAB2IP in the cytoplasm. Interfering with such interaction reduced aggressiveness of cancer cells in xenografts. This interaction is an unexplored mechanism by which mutant p53 can influence tumor evolution, with implications for our understanding of the complex role of inflammation in cancer.

INTRODUCTION

Inflammation is tightly linked to cancer, but a mechanistic comprehension of this complex relationship remains elusive. In the tumor stroma inflammatory chemokines and cytokines, such as Tumor Necrosis Factor alpha (TNFα), modulate the activity of infiltrating immune cells and also the behavior of cancer cells, influencing tumor growth, dissemination, and response to therapy (Grivennikov et al., 2010). Molecular studies aimed at defining the impact of inflammation on specific tumors could thus provide novel prognostic markers and therapeutic strategies.

Expression of mutant p53 isoforms (mutp53) is another significant factor in cancer development, since tumor-associated p53 mutants can actively promote transformation through oncogenic gain-of-function (GOF) activities (Muller and Vousden, 2013; Oren and Rotter, 2010; Walerych et al., 2012).

Various evidences suggest that mutp53 oncogenic properties are deeply connected to tumor inflammation (Solomon et al., 2011, 2012; Yeudall et al., 2012). Perhaps the most convincing is a recent study on a mouse model of inflammatory bowel disease, reporting that mutp53 amplifies and sustains the proinflammatory microenvironment, leading to an increase in the rate of transformation (Cooks et al., 2013).

In addition to favoring inflammation-induced cell transformation, mutp53 may have an unexplored role in shaping the response to inflammation of already transformed cancer cells. In fact, a large proportion of tumors undergo p53 mutation at a later stage of transformation, when an inflammatory microenvironment has been established within the growing tumor mass. The recognition and molecular understanding of a potential mutp53 GOF in inflammation-driven tumor progression and metastasis is therefore of primary interest.

In the recent past, we identified 37 novel interactors of p53 family members (Lunardi et al., 2010). Among them, the RasGAP Disabled2 Interacting Protein (DAB2IP), also called ASK1 Interacting Protein (AIP1), stands out for its role in signaling by growth factors and inflammatory cytokines. Modulating the cell response to multiple potentially oncogenic signals, DAB2IP is a bona fide tumor suppressor and is frequently silenced by promoter methylation in aggressive human tumors (Dote et al., 2004; Qiu et al., 2007). Nonetheless, in many tumors DAB2IP is not methylated, and alternative mechanisms might exist to interfere with its functions.

In the present study, we find that DAB2IP can be functionally inactivated by physical interaction with mutant p53 proteins, with implications for the response of cancer cells to inflammatory cytokines.

RESULTS

Mutant p53 Increases the Invasive Behavior of Cancer Cells Exposed to Inflammatory Cytokines and Specifically TNFα

To explore the role of p53 mutation in the response of cancer cells to inflammation, we performed invasion assays with human breast cancer cell lines bearing mutant p53. To mimic the cytokine milieu of the tumor microenvironment, we used culture medium conditioned by LPS-activated murine dendritic cells (dendritic cell-conditioned medium [DCCM]). The cell lines used are metastatic and thus penetrate matrigel, but DCCM caused a significant increase in such behavior. Under these conditions, p53 depletion abrogated the effect of DCCM, indicating that mutp53 is required for this phenotype (Figures 1A, S1A, and S1B available online).

We performed the same experiments with Ras-trasformed embryonic fibroblasts (MEFs) derived from p53 knockout or p53(R172H) knockin mice; DCCM triggered a greater increase in invasion in mutp53 than in p53 null MEFs (Figure S1D), indicating that this phenotype represents a mutp53 gain of function.

Activated dendritic cells secrete a variety of cytokines and growth factors, including TNFα and TGFβ (Moustakas et al., 2002). Mutant p53 can form a complex with activated Smad2 and p63 in cancer cells, driving metastasis in response to TGFβ (Adorno et al., 2009); a specific TGFβ inhibitor had no impact on the proinvasive response to DCCM, thus excluding this aspect of the mutp53/p63 axis in the phenotype. In contrast, a TNFα blocking antibody efficiently suppressed the increase in invasion induced by DCCM (Figure 1B), indicating that TNFα is the main mediator of this effect.

Mutant p53 Increases Cell Invasion and Protects from Apoptosis in Response to TNFα

We thus repeated the above experiments using recombinant human TNFα, confirming that TNFα increases cell invasion in a mutp53-dependent manner (Figures 1C and S1C). This result was confirmed in a pancreatic cancer cell line with the p53(R273H) mutation (PANC-1), so it is not restricted to mammary cells (Figure S7A).

To verify whether mutp53 is sufficient for this phenotype, we introduced mutant p53 in a nontransformed mammary epithelial cell line. We used MCF-10A stably silenced for endogenous wild-type (WT) p53 (MCF-10A shp53) to generate a panel of mutp53 knockin cell lines (Figure 1D). Notably, TNFα treatment rendered mutp53 knockin cells significantly more invasive than p53 knockdown cells (Figures 1E and S1E). We conclude that mutp53 is sufficient to mediate this response in nontransformed cells.

We also analyzed the death-inducing effects of TNFα on breast cancer cell lines before and after mutant p53 knockdown. With the exception of MDA-MB-231, which underwent a partial G2 arrest (Figure S1F), mutp53 depletion sensitized cells to TNF-induced cell death (Figures 1F and 1G), indicating that mutant p53 restrains TNF-induced activation of proapoptotic pathways. This effect was confirmed also in PANC-1 cells (Figure S7B).

Mutant p53 Modulates TNF-Induced Transcription toward a Proinvasive and Immunogenic Gene Expression Program

To gain a molecular insight on this phenotype, we used microarrays to analyze the transcriptional profile of MDA-MB-231 treated with TNFα for 20 hr, with or without mutp53 silencing. Mutp53 depletion had a broad impact on transcription, independently of TNFα (Figure S2A), so we examined TNF-responsive genes, defined as the union of genes up- or downregulated by TNFα in control and/or mutp53-depleted cells (Table S1). Gene ontology (GO) enrichment analysis revealed that TNF-repressible genes are mostly related to the mitotic cell cycle. In contrast, TNF-inducible genes display a much broader spectrum of biological functions, including cell migration, apoptosis, and inflammatory and immune response (Table S2). Since in our cell models mutant p53 sustains invasion and survival, we focused our analysis on TNF-inducible genes and identified those less expressed (TNF-upsiC) or more expressed (TNF-upsip53) after mutp53 knockdown (Figure 2A).

Microarrays were validated by RT-qPCR on a selection of genes belonging to the TNF-upsiC and TNF-upsip53 groups, confirming their mutp53-dependent regulation (Figures 2C–2D and S2B). We also analyzed public breast cancer data sets with information on p53 status (Curtis et al., 2012; Miller et al., 2011) and verified that the TNF-upsiC metagene is more expressed in tumors with mutp53 (Figure S2C).

We noticed a specific enrichment for the processes "immune response," "cytokine production," and "positive regulation of cell migration" in TNF-upsiC genes (Figure 2B), suggesting that mutp53 modulates a transcriptional response to TNFα that can be proinvasive but also immunogenic. Indeed, TNF-upsiC genes include secreted proteins that can stimulate cell migration and metastasis (e.g., CA12, MMP9, and CXCL10) (Bjordahl et al., 2013; Muthuswamy et al., 2012; Xin et al., 2005), but also chemokines that may control recruitment of lymphocytes (e.g., CXCL10, CX3CL1, and LTB) (Duffy et al., 2000; Hsieh et al., 2010; Shin et al., 2010). To verify this last point, we asked whether medium conditioned by TNFα-treated MDA-MB-231 cells could attract lymphocytes from peripheral blood. We observed chemotaxis of T lymphocytes, cytotoxic T lymphocytes (CTL), and Natural Killer cells. Importantly, this activity was reduced by mutp53 knockdown (Figure S2D). Therefore, mutp53 can affect both cell-autonomous and non-cell-autonomous responses to TNFα.

Mutant p53 Coordinately Modulates TNF-Dependent Activation of Both NF-κB and JNK Pathways

Many of the genes identified are direct NF-κB targets (Table S1), yet they display a striking difference in mutp53 dependency. In addition to NF-κB, TNFα can trigger activation of ASK1/JNK kinases, and these two signaling axes have a complex reciprocal interaction that can affect gene expression (Wajant et al., 2003). We used inhibitors to selectively block either branch of the pathway and analyzed TNF-responsive genes differently affected by mutp53: MMP9 (TNF-upsiC group) and IL1B (TNF-upsip53 group). Not surprisingly, induction of both genes was impaired by the NF-κB inhibitor BAY (BAY11-7082), confirming that they are NF-κB targets (Figure 2E). However, there were

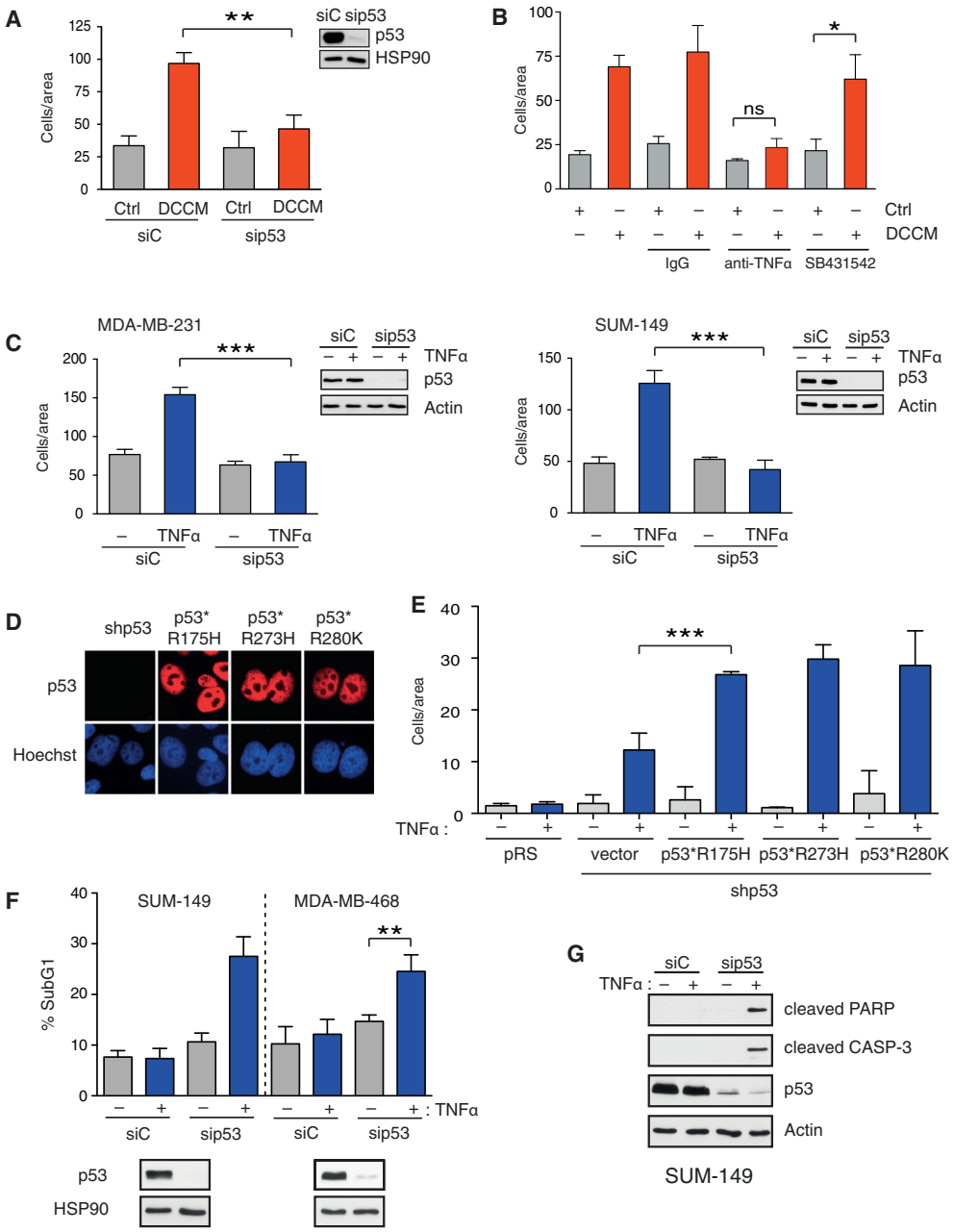

Figure 1. Mutant p53 Amplifies the Aggressive Behavior of Cancer Cells Exposed to Inflammatory Cytokines and TNFα

(A) Mutp53 drives inflammation-induced invasion. MDA-MB-231 cells were transfected with control (siC) or p53 (sip53) siRNAs; invasion assays were performed with medium conditioned by activated dendritic cells (DCCM). Graph summarizes migrated cells per area (mean ± SD; n = 3; **p < 0.01). Mutp53 depletion was checked by western blot. Representative images of migrated cells are shown in Figure S1A.

(B) Invasion assays with MDA-MB-231 were performed as in (A). Rabbit IgG (10 μg/ml), TNFα blocking antibody (10 μg/ml), or TGFβ inhibitor SB431542 (10 μM) were added to the medium as indicated (mean ± SD; n = 3; *p < 0.05).

(C) Invasion assays with indicated cell lines were performed in low serum plus 10 ng/ml TNFα (mean ± SD; n = 3; ***p < 0.001). Representative images of migrated cells are shown in Figure S1C.

(D and E) Mutp53 is sufficient for TNF-induced invasion. MCF-10A cells stably silenced for endogenous WT p53 (shp53) were infected with retroviruses expressing shRNA-resistant (*) p53 mutants. (D) Immunofluorescence of exogenous p53 mutants. Immunoblots are shown in Figure S1E. (E) Transwell migration assays (mean ± SD; n = 3; ***p < 0.001).

(F and G) Mutp53 depletion sensitizes cancer cells to TNF-induced cell death. (F) Indicated cells were treated with TNFα (10 ng/ml) for 48 hr. DNA content was analyzed by flow cytometry (mean ± SD; n = 3; **p < 0.01). Mutp53 depletion was checked by immunoblotting (bottom). (G) SUM-149 cells were treated with TNFα for 24 hr and analyzed by western blot to detect cleaved PARP and CASP-3.

See also Figure S1.

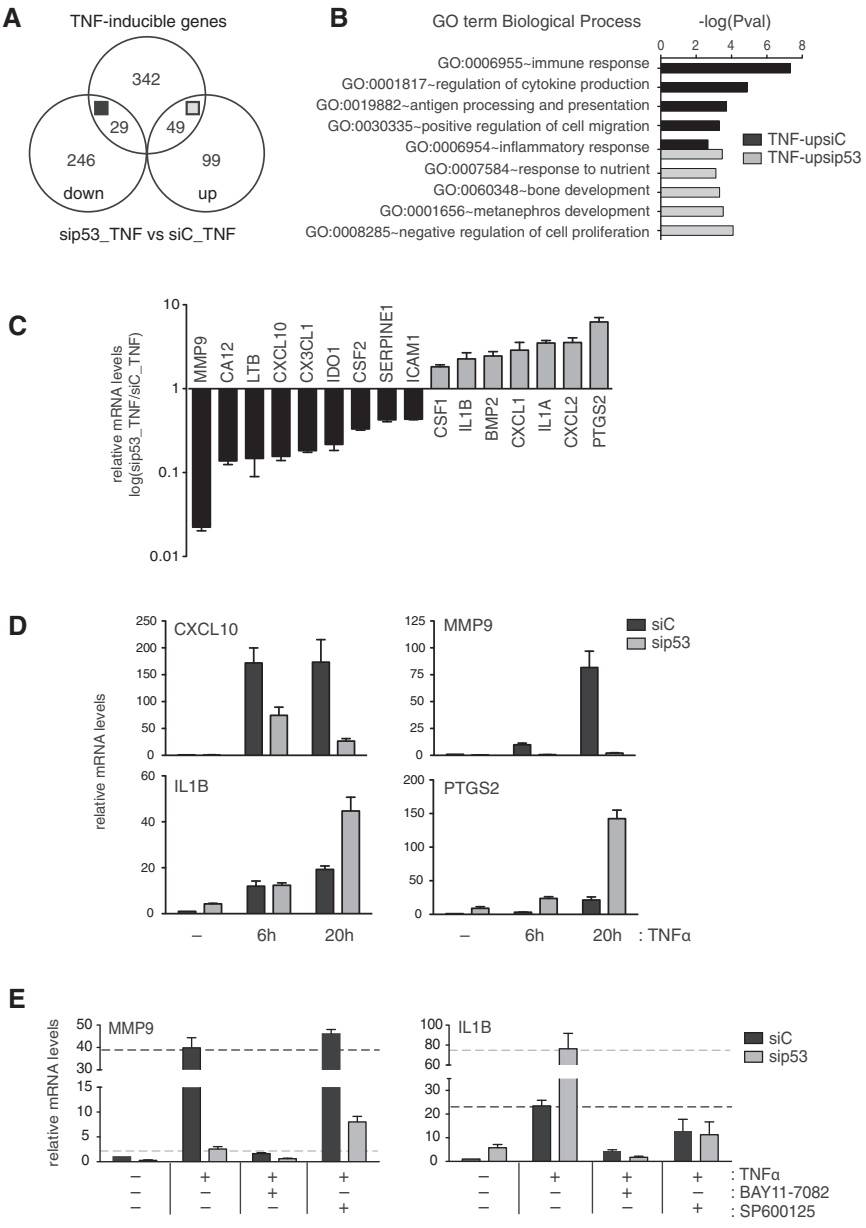

Figure 2. Mutant p53 Modulates the TNF-Induced Transcriptional Landscape

(A) MDA-MB-231 cells were treated with TNFα for 20 hr and analyzed by Illumina microarray. TNF-inducible genes less expressed (TNF-upsiC, black) or more expressed (TNF-upsip53, gray) in mutp53-depleted cells were selected. See also Table S1.

(B) Representative Gene Ontology terms specifically enriched in TNF-upsiC or TNF-upsip53 genes. See also Table S2.

(C) Expression of selected TNF-upsiC (black) or TNF-upsip53 (gray) genes was validated by RT-qPCR. Bars indicate differential expression in sip53_TNF versus siC_TNF samples (mean ± SD; n = 3). Values are normalized to histone H3 and compared to untreated siC-transfected samples.

(D) Expression of selected genes at 6 hr or 20 hr of TNFα treatment. Corresponding p53 immunoblot is shown in Figure S2B.

(E) Mutp53 sustains NF-κB and counteracts JNK-dependent transcription. MDA-MB-231 cells were stimulated with TNFα for 20 hr and treated with the NF-κB inhibitor BAY11-7082 (1 μM) or the JNK inhibitor SP600125 (10 μM) as indicated. Expression of MMP9 and IL1B mRNAs was measured by RT-qPCR as in (C). Dashed lines mark TNF-induced expression levels without inhibitors. See also Figure S2.

interesting differences in the response to the JNK inhibitor SP (SP600125). TNF-dependent induction of IL1B was reduced by SP treatment, indicating that both NF-κB and JNK positively regulate this gene. In contrast, induction of MMP9 was unaffected by SP in control cells but was amplified by SP in p53-depleted cells, suggesting that JNK represses this gene in the absence of mutp53. Identical results were obtained with CXCL10 and PTGS2 (Figure S2E). Transient knockdown of p65 RelA or JNK by siRNA produced similar outcomes (Figure S2F), confirming the direct involvement of these factors.

Together, the data are consistent with a model in which mutp53 sustains activation of NF-κB while counteracts activation of JNK in response to TNFα (Figure S2G). In line with this concept, predicted JUN/AP-1 consensus elements are enriched in the promoters of genes selectively upregulated by TNFα in mutp53-depleted cells (Figure S2H).

Mutant p53 Interacts with the Tumor Suppressor DAB2IP in the Cytoplasm

To unveil the mechanism of this mutp53 action, we searched for a potential target with a role in TNF signaling. We focused on the tumor suppressor DAB2IP, a protein that promotes activation of ASK1/JNK and inhibits activation of NF-κB in response to TNFα (Zhang et al., 2003, 2004).

We had previously found that DAB2IP can bind p53 family proteins (Lunardi et al., 2010); using coaffinity purification assays, we verified that DAB2IP can interact with hot-spot missense p53 mutants, with no evident bias for contact or conformational alterations (Figure 3A). Using deletion constructs, we mapped the reciprocal binding to the C2 domain of DAB2IP and the core domain of different p53 mutants (Figures 3B–3C and Figures S3A and S3B). This suggests that interaction does not involve p53 structural elements required for binding to DNA. This also implies that most p53 mutants can potentially bind DAB2IP.

DAB2IP is a cytoplasmic protein, so interaction with mutp53 must occur in the cytosol. Using fractionation, we verified that mutp53 is present in sizeable amounts in the cytoplasm of metastatic breast cancer cell lines (Figures 3D and S3F). The same was observed with p53 mutants expressed in nontransformed

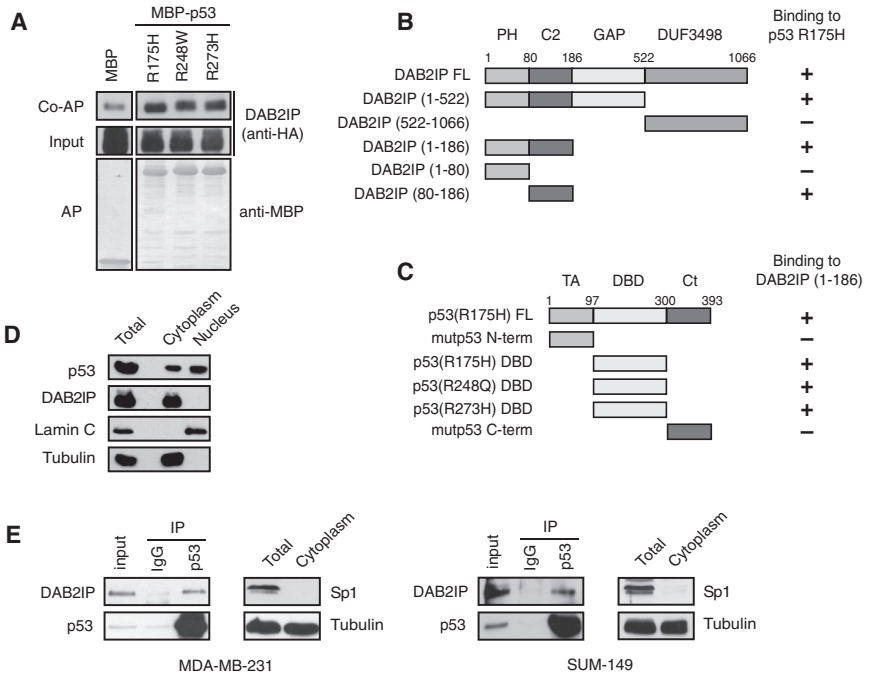

Figure 3. Mutant p53 Interacts with DAB2IP in the Cytoplasm

(A) DAB2IP binds hot-spot p53 mutants. We co-transfected 293T cells with HA-DAB2IP together with plasmids expressing p53 mutants fused to MBP. After purification on amylose resin, HA-DAB2IP bound to mutp53 was detected by western blot (top). Middle: DAB2IP in the lysate (1/40 inputs). Both panels are from the same autoradiography film (i.e., have the same exposure). Bottom: MBP-p53 fusion proteins (baits) after affinity purification.

(B) DAB2IP binds mutp53 through the C2 domain. Schematic representation of DAB2IP deletions used (PH, pleckstrin homology domain; C2, synGAP-like C2 domain; GAP, Ras-GTPase activating domain; DUF3498, domain of unknown function). Binding to MBP-p53(R175H) is indicated.

(C) p53 mutants bind DAB2IP through the DNA binding domain (DBD). Schematic representation of mutant p53 deletions used (TA, transactivation; Ct, oligomerization/C-terminal regulatory). Their interaction with HA-DAB2IP(1-186) is indicated.

(D) Cytoplasmic localization of mutant p53. MDA-MB-231 cells were subjected to fractionation to separate cytoplasm and nuclei. Equal fractions of each sample were analyzed by western blot. Tubulin (cytoplasmic) and Lamin C (nuclear) were blotted as controls.

(E) Endogenous p53(K280R) and p53(M237I) mutants were immunoprecipitated respectively from the cytoplasmic fraction of MDA-MB-231 and SUM-149 cells. Coprecipitated endogenous DAB2IP was detected by western blot. Sp1 was blotted to confirm cytoplasm purity. Input is 1/100 of the cytoplasmic fraction. See also Figure S3.

cells (Figure S3D). Using coimmunoprecipitation, we proved interaction between endogenous DAB2IP and mutp53 in the cytosolic fraction of MDA-MB-231, SUM-149, and PANC-1 cell lines (Figures 3E, S3F, and S7C), with different p53 mutants. No interaction was detected between endogenous DAB2IP and p53 in the cytosolic fraction of cells with wild-type p53 (Figure S3E). Thus, mutant p53 could affect TNF-induced activation of NF-κB and JNK by binding DAB2IP and interfering with its functions.

Inhibition of DAB2IP Defines a Cytoplasmic Gain of Function of mutp53

The above hypothesis implies that mutp53 should affect upstream events of the TNFα transduction cascade (Zhang et al., 2004). We analyzed TNF-induced IKBα and JNK phosphorylation as readouts of NF-κB and ASK1 signaling branches, respectively. In the absence of mutp53, phosphorylation and turnover of IKBα were significantly reduced, indicating less efficient activation (Figures 4A and S7D). Accordingly, nuclear translocation and activation of p65 RelA was also reduced (Figures 4C–4D and S3G).

Depletion of WT p53 had no impact on TNF-induced IKBα phosphorylation and p65 nuclear translocation in HCT116 cells (Figures S3H and S3I), supporting the notion that this phenotype is specific for mutant p53.

Oppositely, phosphorylation of JNK was increased and longer lasting in the absence of mutp53 (Figure 4B), as was phosphorylation of p38 MAPK, another ASK1 downstream kinase (Figure S3J). Together, these effects are consistent with mutp53 inhibiting DAB2IP and provide a mechanistic explanation for the coordinated impact of mutp53 on TNF-induced NF-κB and JNK activation.

The above hypothesis also implies that mutant p53 nuclear activities should not be required for the phenotype. To test this, we replaced the endogenous mutp53 of MDA-MB-231 cells with the same p53 mutant (R280K) deprived of nuclear localization signals (NLSs). Strikingly, expression of mutp53(ΔNLS) efficiently restored TNF-induced invasion in mutp53-depleted MDA-MB-231 cells (Figure 4E). The same was recapitulated in MCF-10A nontransformed cells; replacement of endogenous WT p53 with a cytoplasmic p53 mutant was sufficient to amplify TNF-induced migration (Figure 4F).

We conclude that this specific GOF of mutant p53, i.e., increased invasion triggered by inflammatory cytokines, is largely mediated by its cytoplasmic fraction.

The Effects of mutp53 in Modifying the Response to TNFα Depend on Its Binding to DAB2IP

To assess their functional relationship, we tested whether depletion of DAB2IP could rescue the effects of mutp53 knockdown in our cell models.

Silencing of DAB2IP restored TNF-induced invasion in mutp53-depleted MDA-MB-231 cells (Figures 5A and S4A). Similarly, DAB2IP knockdown protected mutp53-depleted SUM-149 cells from TNF-induced apoptosis (Figure 5B). The same relationship was confirmed in modulation of TNF-dependent

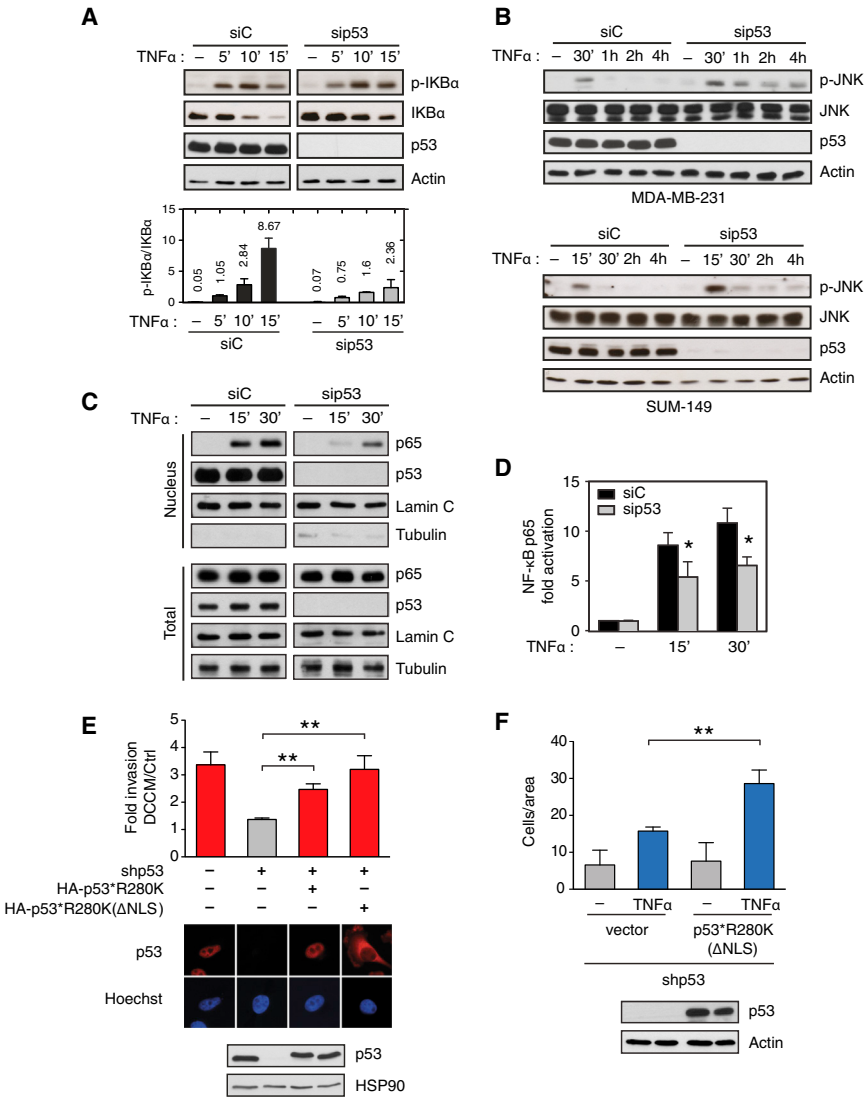

Figure 4. Cytoplasmic Gain of Function of Mutant p53 in the Response to TNFα

(A) Mutant p53 sustains TNF-induced phosphorylation and degradation of IKBα. MDA-MB-231 cells were silenced with indicated siRNAs, serum starved, and treated with TNFα (10 ng/ml). Phosphorylated and total IKBα were measured by immunoblotting. Bottom: the ratio of phosphorylated over total IKBα measured by densitometry on autoradiography films (mean ± SD; n = 3).

(B) Depletion of mutant p53 increases JNK activation by TNFα. Cells were treated as in (A). Phosphorylated and total JNK were analyzed by immunoblotting.

(C and D) Mutant p53 sustains TNF-induced nuclear translocation and activation of p65 RelA. (C) Cells were treated as in (A). At the indicated times cells were subjected to biochemical fractionation to separate cytoplasm and nuclei. Tubulin (cytoplasmic) and Lamin C (nuclear) were blotted as controls. (D) Sequence-specific DNA binding of p65 RelA was measured by ELISA in cells treated as in (A) (mean ± SEM; n = 4; *p < 0.02). Control immunoblots are shown in Figure S3G.

(E and F) Cytoplasmic mutant p53 promotes inflammation-driven invasion. (E) MDA-MB-231 cells stably silenced for mutp53 (shp53) were infected with retroviruses encoding shRNA-resistant (*) versions of p53(R280K) or its cytoplasmic variant p53(R280K)ΔNLS. Matrigel invasion assays with DCCM were done as in Figure 1A. Bars indicate the fold increase in migrated cells in DCCM versus Ctrl medium (mean ± SD; n = 3; **p < 0.01). Localization of mutp53 proteins (red) was verified by immunofluorescence. Expression levels were checked by western blot (bottom). (F) MCF-10A cells stably silenced for WT p53 (shp53) were infected with p53(R280K)ΔNLS retrovirus as in (E). Transwell migration assays with TNFα were performed as in Figure 1E (mean ± SD; n = 3; **p < 0.01). Expression of p53 was checked by western blot (bottom).

See also Figure S3.

transcription, whereby knockdown of DAB2IP strikingly reverted the effects of mutp53 depletion (Figures 5C, S4B, and S7E).

Finally, depletion of DAB2IP restored to normal the kinetics of TNF-induced IKBα, JNK, and p38 phosphorylation (Figures 5D–5F and S4C), as well as p65 nuclear translocation (Figure 5E), in mutp53 knockdown cells. In all these experiments, depletion of DAB2IP had a negligible impact on TNF-induced phenotypes in the presence of mutp53, strongly supporting an epistatic relationship between the two proteins.

Consistently, DAB2IP overexpression abolished the proinvasive behavior of MDA-MB-231 cells in response to inflammatory cytokines. Of note, escalating mutp53 levels in the cytoplasm, by overexpression of p53(R280K)ΔNLS, fully restored inflammation-induced invasion in DAB2IP-overexpressing cells (Figure 6A), strongly suggesting that mutp53 interferes with DAB2IP metastasis-suppressive functions.

DAB2IP interaction with the protein kinase ASK1 is required to funnel TNF-signaling toward the JNK pathway (Min et al., 2008; Zhang et al., 2003, 2004). In transient overexpression, we verified that increasing amounts of p53(R280K) caused a progressive reduction in the fraction of DAB2IP bound to ASK1 (Figures 6B and S5A). Analyzing endogenous proteins, we observed that depletion of mutant p53 in MDA-MB-231 cells increased the fraction of DAB2IP coimmunoprecipitated with ASK1 (Figure 6C). Together, these data indicate that mutp53 can compete with DAB2IP physiological interactors and in particular ASK1.

Disruption of the mutp53-DAB2IP Interaction Reduces the Invasive Behavior of Cancer Cells Exposed to Inflammation

We found that mutp53 binds to the N terminus of DAB2IP. To interfere with this interaction, we constructed a chimeric protein in which the first 186 amino acids of DAB2IP are fused to GFP. Since the C2 domain of DAB2IP is also involved in interaction with ASK1, we introduced a mutation that specifically prevents ASK1 binding (KA2, Figures S5B and S5C) (Zhang et al., 2003).

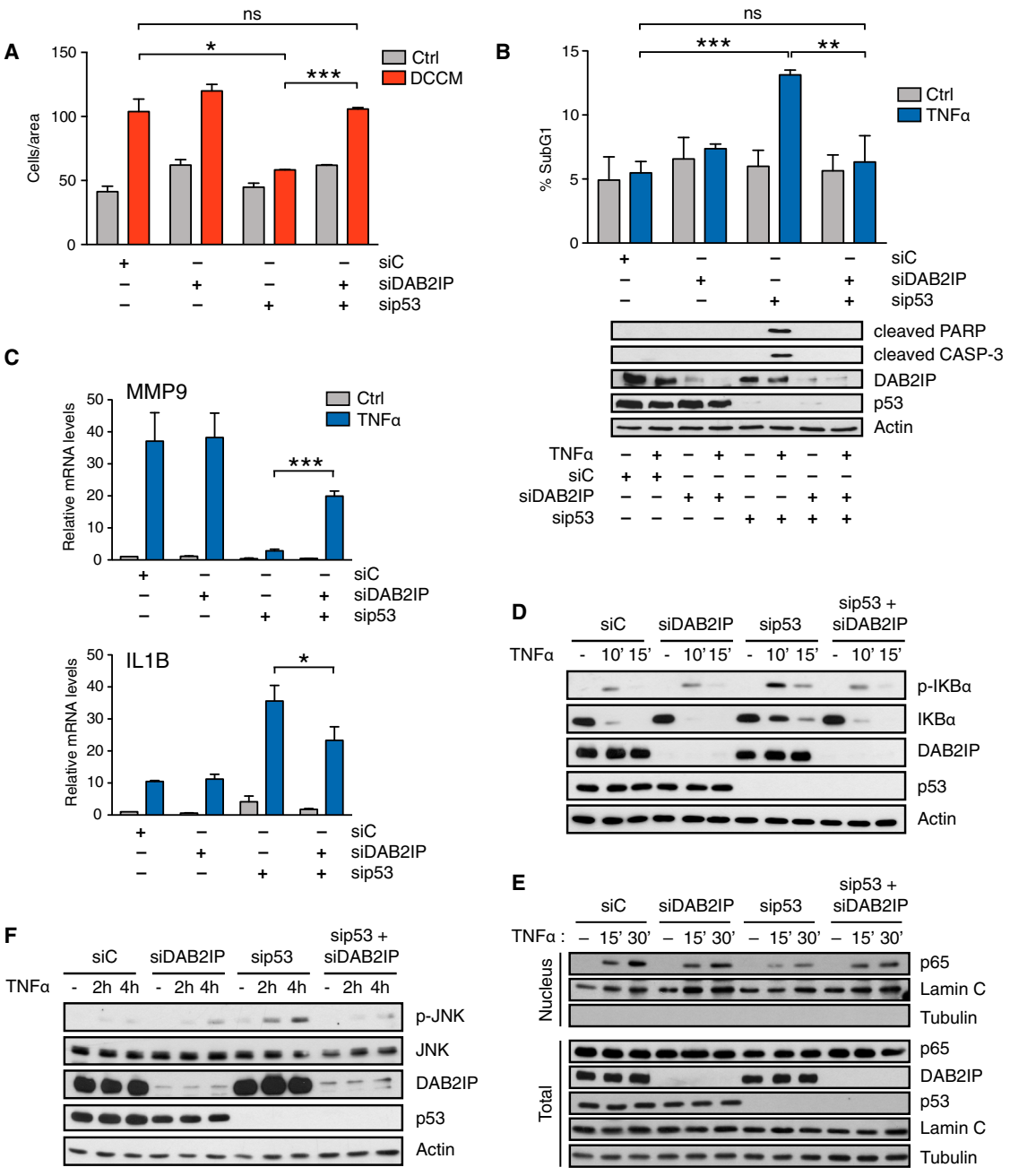

Figure 5. Functional Interaction between Mutant p53 and DAB2IP in the Response of Cancer Cells to Inflammatory Cytokines

(A) MDA-MB-231 were silenced for DAB2IP and/or mutp53 as indicated. Invasion assays were performed with DCCM as in Figure 1A (mean ± SD; n = 2; *p < 0.05; ***p < 0.001). Control immunoblot in Figure S4A.

(B) SUM-149 were silenced for DAB2IP and/or mutp53, serum starved, and treated with TNFα (10 ng/ml) for 24 hr. The fraction of cells in SubG1 was measured by flow cytometry (mean ± SD; n = 3; **p < 0.01; ***p < 0.001). The same cells were analyzed by western blot to detect cleaved PARP and CASP-3 (bottom).

(C) MDA-MB-231 were silenced for DAB2IP and/or mutp53. Expression of MMP9 and IL1B was measured by RT-qPCR after 20 hr of TNFα as in Figure 2 (mean ± SD; n = 3; *p < 0.05; ***p < 0.001). Control immunoblot in Figure S4B.

(D) MDA-MB-231 were silenced for DAB2IP and/or mutp53, and treated with TNFα as in Figure 4A. Phosphorylated and total IKBα were detected by immunoblotting.

(E) Cells treated as in (D) were subjected to biochemical fractionation to separate cytoplasm and nuclei. Tubulin (cytoplasmic) and Lamin C (nuclear) were blotted as controls.

(F) MDA-MB-231 were silenced for DAB2IP and/or mutp53 and treated with TNFα as in (D). Phosphorylated and total JNK were analyzed by immunoblotting. See also Figure S4.

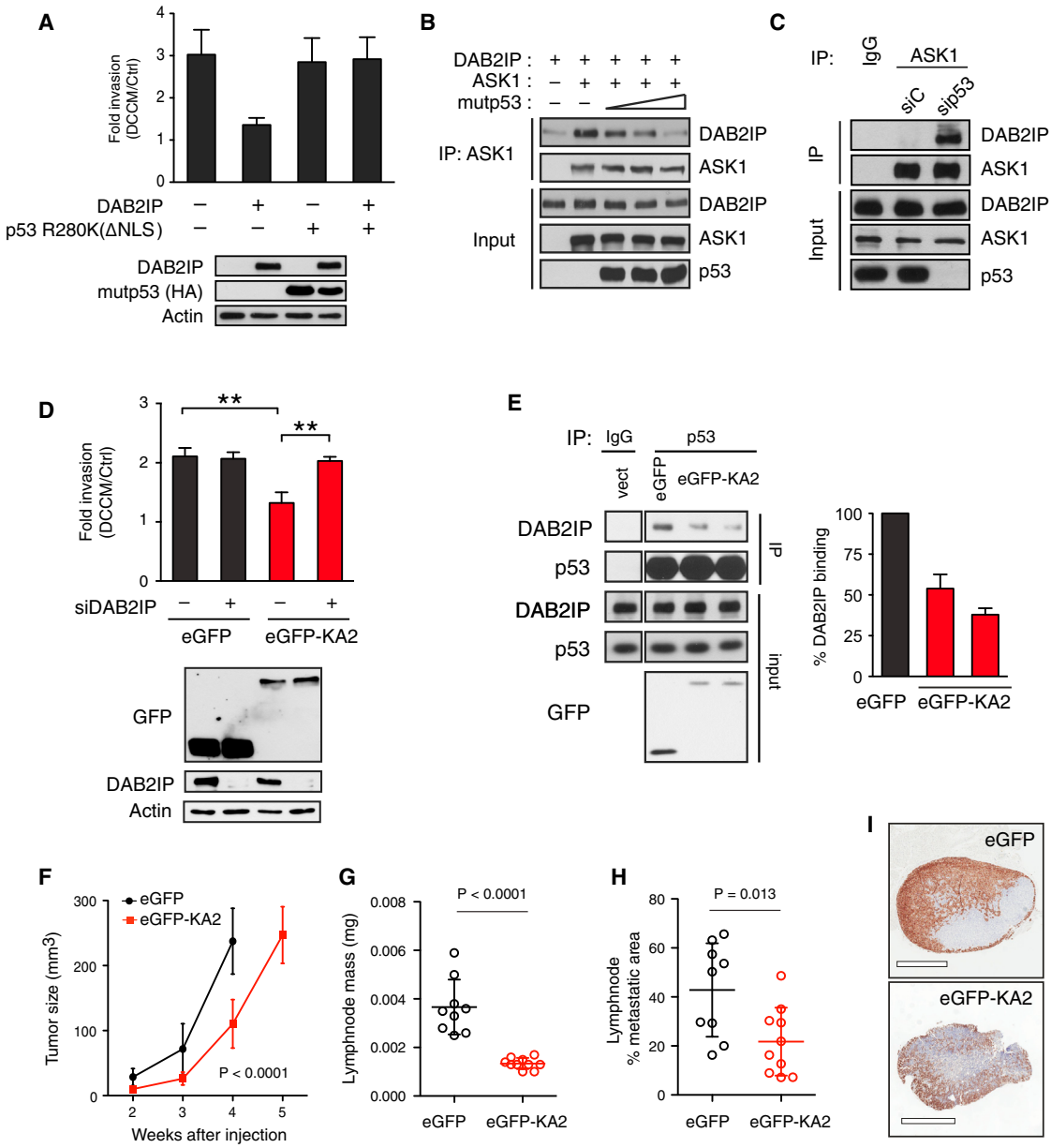

Figure 6. The mutp53-DAB2IP Interaction Is Required for the Invasive Response of Cancer Cells to Inflammation

(A) MDA-MB-231 cells stably overexpressing DAB2IP were infected with a retrovirus expressing HA-p53(R280K)ΔNLS or an empty retrovirus as a control. Invasion assays with DCCM were done as in Figure 1A; bars indicate the increase in cells migrated in DCCM versus Ctrl medium (mean ± SD; n = 3). Protein levels were checked by western blot (bottom).

(B) H1299 cells were cotransfected with plasmids expressing DAB2IP, HA-ASK1, and increasing amounts of p53(R280K). The fraction of DAB2IP bound to ASK1 was analyzed by western blot of ASK1 immunoprecipitates. Quantification is reported in Figure S5A.

(C) MDA-MB-231 cells were transfected with control or p53 siRNA. Endogenous DAB2IP bound to ASK1 was analyzed by coimmunoprecipitation with anti-ASK1 antibody.

(D) MDA-MB-231 cells were stably transduced with retroviruses expressing eGFP-DAB2IP$_{1\text{-}186}$KA2 fusion protein (eGFP-KA2) or eGFP alone, with or without DAB2IP siRNA as indicated. Invasion assays were performed as in (A) (**p < 0.01). Expression of eGFP proteins and endogenous DAB2IP was analyzed by western blot (bottom).

(E) Endogenous mutp53 was immunoprecipitated from MDA-MB-231 cells stably expressing eGFP or eGFP-KA2 (two lines derived from independent infections); coprecipitated endogenous DAB2IP was analyzed by western blot. Specificity was checked using unrelated IgG (panels are from the same autoradiography). The fraction of DAB2IP bound to mutp53 was quantified by densitometry; bars represents the DAB2IP$_{(co\text{-}IP)}$/DAB2IP$_{(input)}$ ratio, normalized to the eGFP sample (mean ± SD; n = 3).

(F–I) Expression of DAB2IP$_{1\text{-}186}$KA2 reduces growth and dissemination of cancer cells in immunocompromised mice. MDA-MB-231 cells stably expressing eGFP or eGFP-KA2 were injected in the mammary fat pad of SCID mice and different parameters of tumor progression were evaluated. (F) Kinetics of primary tumor

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Invasion assays were performed with MDA-MB-231 expressing the eGFP-DAB2IP(1-186)KA2 fusion protein. The KA2 decoy protein had no obvious effects on cell growth and motility, but clearly reduced invasion triggered by inflammatory cytokines; notably, this effect requires DAB2IP (Figures 6D and S5D). The inhibitory action of the KA2 peptide was also verified in PANC-1 cells (Figure S7F).

Mechanistically, overexpression of the KA2 decoy significantly diminished the amount of endogenous DAB2IP coimmunoprecipitated with mutp53 (Figure 6E), confirming that reduced invasion might be due to an increase in "free" DAB2IP protein.

We next extended these experiments to an in vivo model. When injected orthotopically in the fat pad of nude mice, MDA-MB-231 cells expressing the KA2 fusion protein formed smaller tumors than control eGFP-expressing cells (Figure 6F), indicating reduced aggressiveness. To evaluate metastatic dissemination, homolateral axillary lymph nodes were collected and screened for invading human cells when the primary tumors reached a similar size. Gross weight and immunohistochemistry (Figures 6G–6I and S5E) confirmed that KA2-expressing cells were less abundant in lymph nodes than eGFP-expressing controls, clearly indicating reduced metastasis. Importantly, analysis of primary tumors confirmed that mutp53 levels were not affected (Figure S5F).

Implications of the mutp53/DAB2IP Interaction in Breast Cancer: A Meta-analysis

To explore the potential clinical impact of this molecular axis, we analyzed public gene expression data sets. We focused on basal-like triple negative breast cancers (TNBCs) that have a high frequency of p53 mutation and are an outstanding challenge for clinical management (Carey et al., 2010; Foulkes et al., 2010; Walerych et al., 2012).

We used a large metadata set of 19 independent studies, consisting of 3,254 individuals (see Supplemental Information) (Rustighi et al., 2014), and stratified patients based on expression of the mutp53-dependent TNF-inducible genes that were identified in MDA-MB-231 cells (Figure 2). To minimize cell line bias, we selected a subset of these genes consistently coexpressed in mutp53-enriched breast cancers (upsiC10, Figure S6A). The upsiC10 signature was not prognostic on the entire data set, but correlated with prolonged disease-free survival (DFS) in basal/TNBC patients (Figure 7A). We next restricted our analysis to data sets with information on p53 status. Notably, the upsiC10 metagene was predictive of survival specifically in patients with mutant p53 (Figure 7B). Similar results were obtained using all genes in the TNF-upsiC group (Figure S6B and Table S3).

Since mutp53 makes cancer cells more invasive in response to inflammation in vitro, the improved patients' survival suggests a host interaction effect. We noticed that genes associated to the TNFα/mutp53 axis include powerful chemokines that can promote recruitment of immune cells (Figures 2 and S2D), and an increased B and T cell infiltrate has been correlated to prolonged DFS in breast cancer (Finak et al., 2008; Fridman et al., 2012). We thus analyzed expression of immune-specific gene signatures as a proxy to infer amount and composition of infiltrating lymphocytes in tumor samples. We used previously published metagenes already validated in colon (Bindea et al., 2013) and breast cancer (Rody et al., 2009, 2011).

Within our cohort of basal breast cancers, lymphocyte metagenes were significantly more expressed in tumors with higher expression of the upsiC10 signature (Figures 7C and S6C), indicative of increased infiltration. Intriguingly, we found higher expression of cytotoxic (CTL+NK) and Th1, but not Th2, metagenes, suggesting that infiltrating T cells might be preferentially polarized toward a proinflammatory phenotype.

In line with studies by others (Rody et al., 2009, 2011), high expression of immune metagenes (with the remarkable exception of Th2 cells) correlated with prolonged DFS in basal/TNBC patients (Figures 7D and S6D). Strikingly, expression of immune metagenes correlated with better DFS specifically in tumors with mutant p53 (Figure 7E and Table S3).

Together, these observations suggest that mutp53 reprograms inflammatory signaling toward a gene expression profile that increases cell motility and survival but also promotes activation of tumor immune cells, potentially affecting clinical outcome.

DISCUSSION

Various evidences, including our work, indicate that mutation of p53 plays a primary role in the relationship between inflammation and cancer, suggesting that mutp53 may shift the effects of inflammation toward oncogenic outcomes. A recent study showed that p53 missense mutation can be a driver in inflammation-induced cancer formation (Cooks et al., 2013). Here, we demonstrate that mutp53 can also drive inflammation-induced cancer progression.

We observed that mutant p53 can influence multiple downstream molecular pathways activated by the TNF receptor. While confirming that mutp53 sustains TNF-induced activation of NF-κB, as previously reported by others (Cooks et al., 2013; Schneider et al., 2010; Weisz et al., 2007), we demonstrated that mutp53 also dims TNF-induced activation of ASK1/JNK. This dual effect is translated in a characteristic gene expression profile, with strong activation of a group of NF-κB target genes, but reduced or no activation of another group of NF-κB target genes.

Previous studies reported that mutp53 is recruited to κB sites on the promoters of inflammatory genes in response to TNFα (Cooks et al., 2013; Schneider et al., 2010; Weisz et al., 2007). We discovered that mutp53 also acts upstream, shifting the balance between activation of the NF-κB and ASK1/JNK pathways in response to TNFα. Mechanistically, a fraction of

growth. eGFP, black (n = 10); eGFP-KA2, red (n = 10). (G) Weight of homolateral lymph nodes when primary tumors reached similar size. eGFP, black, 4 weeks (n = 9); eGFP-KA2, red, 5 weeks (n = 10). (H) Percentage of area occupied by invading cells in lymph nodes, quantified by immunohistochemistry of human cytokeratin. (I) Representative sections of lymph nodes stained for human cytokeratin (bar = 1 mm).
See also Figure S5.

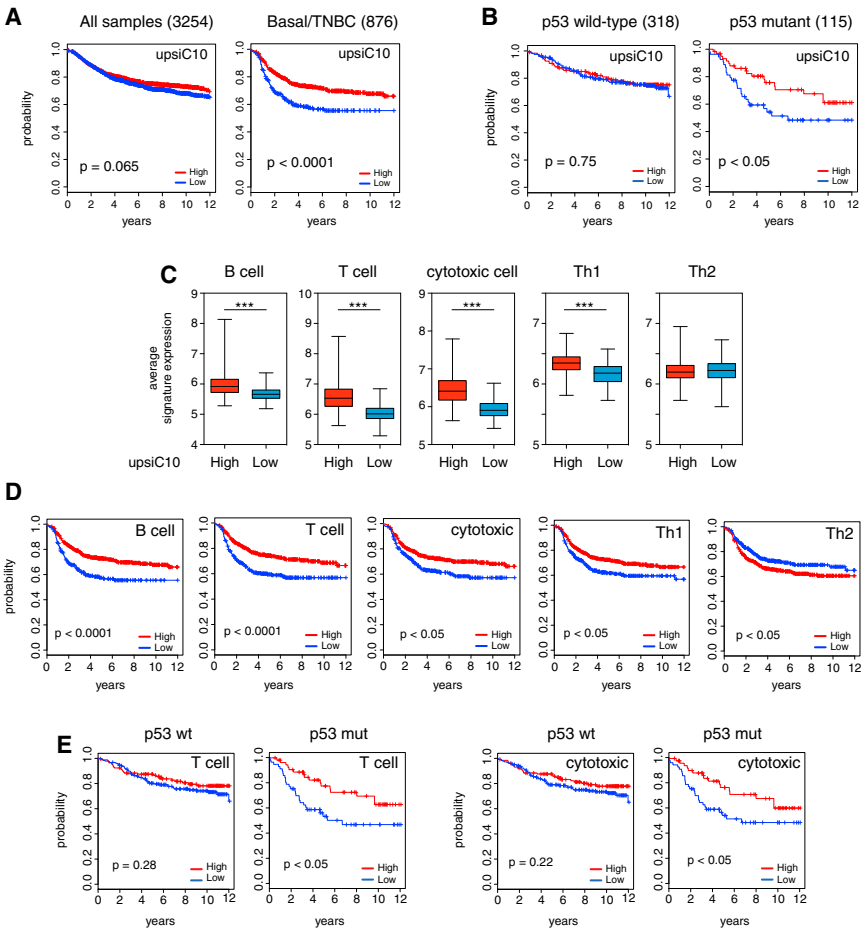

Figure 7. In Silico Analysis Reveals a Potential Impact of the TNF/mutp53 Axis in the Clinical Outcome of Breast Cancer

(A) Kaplan-Meier (K-M) survival curves for high or low expression of the upsiC10 signature in a large breast cancer metadata set and basal-like/TNBC samples (see Supplemental Information for details).

(B) K-M survival curves for high or low expression of the upsiC10 signature in breast cancers with known p53 status.

(C) Expression of metagenes identifying specific immune cell populations (Bindea et al., 2013) in basal-like/TNBC breast cancers divided according to high or low expression of the upsiC10 signature (***$p < 0.0001$).

(D) K-M survival curves for high or low expression of immune metagenes in basal-like/TNBC breast cancers.

(E) K-M survival curves for high or low expression of T cell and cytotoxic cell metagenes in breast cancers with known p53 status.

See also Figure S6 and Table S3.

mutp53 binds DAB2IP in the cytoplasm, interfering with formation of TNF-induced signaling complexes that activate the ASK1/JNK axis, thereby promoting activation of NF-κB.

DAB2IP is a tumor suppressor protein that is rarely mutated in cancer but is often silenced by promoter methylation (Dote et al., 2004; Qiu et al., 2007). Our data indicate that binding by mutp53 is an alternative means to inactivate DAB2IP functions in cancer. Notably, experiments with nuclear-excluded p53 mutants indicate that this activity may be sufficient to mediate the invasive response triggered by inflammatory cytokines in cancer cells. So, this is a bona fide cytoplasmic gain of function of mutp53.

In addition to funneling TNFα signals toward growth-restraining activation of ASK1, DAB2IP negatively modulates multiple oncogenic signaling pathways: most notably Ras and Akt (Min et al., 2010; Xie et al., 2009, 2010). It is quite possible that the mutp53/DAB2IP interaction in cancer cells may have broader effects than promoting inflammation-induced invasion. For instance, a role of mutant p53 has been described in sustaining Ras (Sauer et al., 2010) as well as Akt activation (Dong et al., 2009), but a convincing mechanism has not been described. It is possible that the mutp53/DAB2IP interaction might partially explain those observations as well.

DAB2IP can bind also WT p53, at least in vitro and in transient overexpression (Lunardi et al., 2010), raising interesting questions on whether such interaction can occur physiologically. In normal conditions, WT p53 concentration in the cytoplasm is very low, and we could not detect binding of the endogenous proteins. Accordingly, WT p53 depletion had no impact on DAB2IP-dependent TNF signaling steps (Figure S3), strongly indicating that the phenotypes described here are peculiar to mutant p53. Nonetheless, it is tempting to speculate that binding with DAB2IP can occur upon specific stress conditions that increase cytoplasmic p53, thus potentially linking p53 to nontranscriptional modulation of multiple signaling pathways. Additional work will be required to explore this possibility and its implications.

Our finding that tumor-derived p53 mutants can bind DAB2IP in the cytoplasm, competing with functionally relevant DAB2IP protein partners, provides striking evidence that mutant p53 can gain oncogenic functions by making nonphysiological interactions with other proteins—not necessarily transcription factors—and interfering with their function (Muller and Vousden, 2013; Oren and Rotter, 2010).

We tested the above principle by designing a fusion protein that can interfere with mutp53-DAB2IP binding. Notably, expression of such decoy abolished TNF-induced invasion in vitro, and xenograft growth and dissemination in vivo, confirming that this interaction is functionally relevant. These results provide a crucial proof of principle that peptide or nucleotide aptamers designed to interfere with the mutp53/DAB2IP interaction might have a potential application in targeted therapy of mutp53 cancers. Future work in this direction is warranted.

Various studies described WT p53 as an important mediator in the crosstalk between inflammatory microenvironment and cell behavior. In a complex interplay with NF-κB, p53 modulates expression of immune-response and inflammation genes; such

secretory phenotypes can contribute to tumor suppression in a non-cell-autonomous manner, for instance, controlling the inflammatory microenvironment and promoting macrophage M1- versus M2-type polarization (Lowe et al., 2013; Lujambio et al., 2013; Schwitalla et al., 2013). Loss of p53 can thus affect inflammatory pathways, eventually contributing to tumor formation.

In this study, we provide compelling evidence that mutation of p53, compared to its loss, confers additional properties to cancer cells, making them more aggressive in response to inflammatory cytokines and in particular TNFα. Intriguingly, our data also indicate that cancer cells with mutant p53 respond to TNFα by expressing secreted molecules that promote recruitment of immune cells, a phenomenon that can be tumor restraining (Fridman et al., 2012; Galon et al., 2013). We think this is a sort of "Achilles' heel" of mutp53 cancers; in response to inflammation, mutp53-modulated activation of NF-κB conveys powerful proinvasive and prosurvival activities but, at the same time, triggers expression of chemokines that can recruit leukocytes and modulate their functions within the tumor. Increased recruitment of B and T cells is associated with longer relapse-free survival in breast and other cancers (Fridman et al., 2012; Lee et al., 2013; Schmidt et al., 2008). Accordingly, our in silico studies indicate that cancers with mutant p53 have a better outcome if they express a gene signature linked to the mutp53/TNFα axis. These observations suggest that a relatively simple standard clinical-pathological parameter such as quantity and quality of infiltrating immune cells—i.e., the immunoscore (Galon et al., 2013)—may improve prediction of the clinical outcome of mammary tumors with mutant p53.

EXPERIMENTAL PROCEDURES

Cell Lines and Treatments

The following human cell lines were used: MDA-MB-231 (p53R280K), MDA-MB-468 (p53R273H), SUM-149 (p53M237I), PANC-1 (p53R273H), MCF-10A (p53 wild-type), HCT116 (p53 wild-type), and U2OS (p53 wild-type).

For gene expression analysis, cells were treated with recombinant human TNFα (10 ng/ml) in low serum (0.1% FBS) for the indicated times. When necessary, JNK (SP600125) and NF-κB (BAY11-7082) inhibitors were added to the medium 2 hr before TNFα. For analysis of IKBα, p38, and JNK phosphorylation, cells were serum starved for 24 hr before TNFα (10 ng/ml) addition.

Migration and Invasion Assays

For transwell migration assays, cells (0.5–1 × 10^5) were plated on 24-well PET inserts (8.0 μm pore size, Falcon). For invasion assays, cells (0.5–1 × 10^5) were plated on 24-well PET inserts (8.0 μm pore size, Falcon) coated with BD Matrigel (BD Bioscience). Cells that passed through the filter were fixed, stained, and counted after 16 hr or 18 hr, respectively. For invasion assays using DCCM, cells were seeded in 10% FBS on the upper chamber, while the lower chamber was filled with DCCM or control medium.

For invasion or transwell assays using recombinant TNFα, cells were pretreated with the cytokine; specifically, cells were kept in low (0.1%) serum with or without TNFα (10 ng/ml) for 24 hr. Subsequently, cells were trypsinized, counted, and reseeded on filters in low serum, with or without TNFα. The lower chamber was filled with high serum medium without TNFα.

Gene Expression Analysis

Total RNA was extracted with QIAzol (QIAGEN). For RT-qPCR, 1 μg of total RNA was reverse transcribed with QuantiTect Reverse Transcription kit (QIAGEN). Real-time PCR was performed using SsoAdvanced SYBR Green Master Mix (Biorad) on a CFX96 Real-Time PCR System (Biorad). Primer sequences are listed in Supplemental Experimental Procedures.

For microarrays, three biological replicates for each group (siC, siC_TNF, sip53, sip53_TNF) were hybridized on HumanHT-12-v4-BeadChip (Illumina). Processing and analysis of the data are described in detail in Supplemental Experimental Procedures.

In Vivo Tumorigenicity and Metastasis Assays

For xenografts, cells (1 × 10^6) were resuspended in 100 μl of DMEM and injected into the mammary fat pad of previously anesthetized 6-week-old SCID female mice. Tumor growth at the injection site was monitored by caliper measurements. For metastasis evaluation, animals were anesthetized and sacrificed at times when tumors were of similar size. Primary tumors were extracted and directly frozen in liquid nitrogen for molecular analyses. Lymph nodes were excised, weighted, formalin fixed, and paraffin embedded for hematoxylin-eosin staining and Cytokeratin 7 immunohistochemistry.

ACCESSION NUMBERS

The MDA-MB-231 expression array experiment was deposited in GEO with accession number GSE53153.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.10.013.

AUTHOR CONTRIBUTIONS

G.D.M. and A.B. designed and performed most experiments and contributed equally to this work. M.D.F., G.C., D.R., and R.S. performed in vitro and in vivo experiments. S.N., S.P., and L.C. did bioinformatics analyses. R.B., A.R., S.B., and G.D.S. supervised experiments and discussed and interpreted results of the study. G.D.M., A.B., and L.C. conceptualized the work and wrote the manuscript. L.C. supervised the project.

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