LETTER

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SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia–inducible factors

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The molecular determinants of malignant cell behaviours in breast cancer remain only partially understood[1]. Here we show that SHARP1 (also known as BHLHE41 or DEC2) is a crucial regulator of the invasive and metastatic phenotype in triple-negative breast cancer (TNBC), one of the most aggressive types of breast cancer. SHARP1 is regulated by the p63 metastasis suppressor and inhibits TNBC aggressiveness through inhibition of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α (HIFs). SHARP1 opposes HIF-dependent TNBC cell migration *in vitro*, and invasive or metastatic behaviours *in vivo*. SHARP1 is required, and sufficient, to limit expression of HIF-target genes. In primary TNBC, endogenous SHARP1 levels are inversely correlated with those of HIF targets. Mechanistically, SHARP1 binds to HIFs and promotes HIF proteasomal degradation by serving as the HIF-presenting factor to the proteasome. This process is independent of pVHL (von Hippel–Lindau tumour suppressor), hypoxia and the ubiquitination machinery. SHARP1 therefore determines the intrinsic instability of HIF proteins to act in parallel to, and cooperate with, oxygen levels. This work sheds light on the mechanisms and pathways by which TNBC acquires invasiveness and metastatic propensity.

Breast cancer is a heterogeneous disease, both biologically and clinically[2]. An important parameter in the clinical management of breast cancer patients is the expression of steroid hormone receptors (oestrogen and progesterone receptors) and ERBB2 (also known as HER2) overexpression and/or amplification; effective tailored therapies have in fact been developed for patients with hormone receptor-positive or HER2-positive diseases[2]. In contrast, TNBC is defined merely by the lack of expression of oestrogen receptor, progesterone receptor and HER2, and thus the category includes tumours that are clinically and pathologically diverse, for which we strive to identify tumour-addicted molecular pathways[1]. Understanding TNBC is of pivotal importance not only in light of the current lack of therapeutic options but also because TNBC accounts for some of the most aggressive types of breast cancers, marked by high rates of relapse, visceral metastases and early death[1].

TAp63 (one of the two main isoforms encoded by *p63*) has recently emerged as a key suppressor of invasive and metastatic cell behaviours in breast cancer, and other tumour types[3–5]; for example, mice that have TAp63 genetically ablated develop aggressive and metastatic carcinomas[4]. We recently identified two genes, *SHARP1* and *CCNG2* (cyclin G2), that are downstream of TAp63α (a sub-isoform of TAp63) in breast cancer cells[3]. To investigate the involvement of this pathway in promoting malignancy and metastatic spread in TNBC, we started by collecting a cohort of 250 primary TNBC samples from eight clinically annotated gene-expression data sets (see Methods and Supplementary Tables 1, 2 and 3), and examined whether the expression of *SHARP1* and *CCNG2* has prognostic value in TNBC. For this, we defined two groups of TNBC with high and low levels of *SHARP1* and *CCNG2* expression, respectively (Supplementary Fig. 1). Crucially, using univariate Kaplan–Meier analyses, individuals in the TNBC group with low *SHARP1* and *CCNG2* expression were shown to have a significantly higher probability of developing metastasis and of reduced survival (Fig. 1a and Supplementary Fig. 1). Moreover, we discriminated between different p63 carboxy-terminal variants using the probe sets present on the TNBC microarrays, and found that the levels of *SHARP1* and *CCNG2* expression correlated with the levels of p63α/β, but not of p63γ (Supplementary Fig. 2).

The fact that expression of just two genes could be prognostic in TNBC indicates that in addition to their utility as markers they may have functional roles in tumour aggressiveness. To gain insights into the mechanisms by which SHARP1 and CCNG2 are linked to malignant progression, we investigated whether their expression could be linked to other known tumorigenic pathways by gene set enrichment analyses (GSEA, see Methods). Specifically, we searched in tumour samples for statistical associations between low *SHARP1* and *CCNG2* expression, and other gene 'signatures' that register elevated activity of various signalling pathways or dysregulated cellular processes, for a total of 254 signatures (Supplementary Tables 5 and 6). Interestingly, signatures of TGFβ activity and p53 mutation were most strongly associated with low *SHARP1* and *CCNG2* expression (Fig. 1b). Hence, clinical data confirm that mutant p53 and TGFβ are both involved in the regulation of *SHARP1* and *CCNG2*, as previously shown in the well-established TNBC cellular model MDA-MB-231 (also known as MDA-231) (ref. 3). We then focused on the signature with the second strongest association with low *SHARP1* and *CCNG2* expression: a signature that denotes high HIF activity (Fig. 1b). HIFs have been involved in several aspects of malignancy in several tumour types[6–8]; in breast cancer, a large body of clinical data shows that high levels of HIF-1α or HIF-2α in tissue samples are linked to poor prognosis and high patient mortality[8,9]. Several results strongly support the role of HIFs in TNBC: first, a signature of HIF activity predicted metastasis proclivity in our cohort of TNBC (Supplementary Fig. 3); second, combined loss of HIF-1α and HIF-2α severely impaired lung colonization of MDA-231 cells injected in the tail vein of immunocompromised mice (Fig. 2a; refs 10, 11); and third, short interfering RNA (siRNA)-mediated depletion of HIF-1α or HIF-2α potently inhibited trans-well migration that is triggered by TGFβ (Supplementary Fig. 4).

We next examined whether SHARP1 or CCNG2 are causal for the repression of HIFs. Interestingly, a physical association between overexpressed HIF-1α and SHARP1 has been previously noted in transfected COS7 cells[12]. We found a robust physical association between HIF-1α and SHARP1 at endogenous levels in independent TNBC cell lines, such as MDA-231, Hs578T and SUM159 (Fig. 1c, data not shown). Conversely, no interaction was detected between HIF-1α and CCNG2 (data not shown); moreover, in contrast with SHARP1 (see below), CCNG2 overexpression had no inhibitory effect on the

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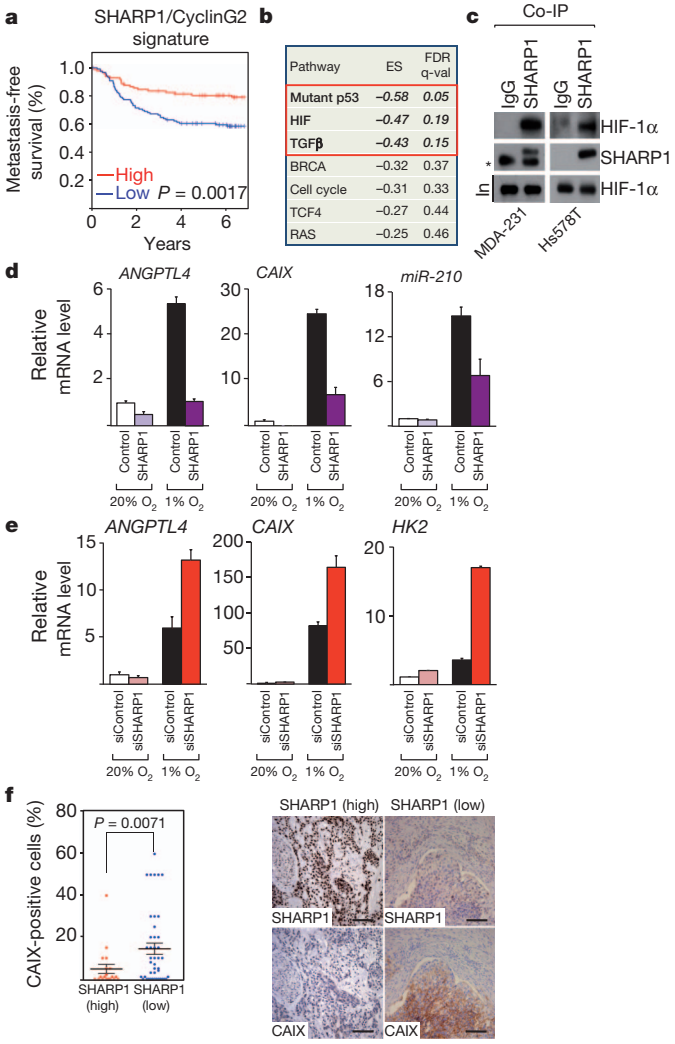

Figure 1 | SHARP1 is an inhibitor of HIF activity. **a**, Kaplan–Meier graph representing the probability of metastasis-free survival in TNBC patients, stratified according to high or low expression levels of *SHARP1* and *CCNG2* (*SHARP1*/*CCNG2* signature). In multivariate analysis, the *SHARP1*/*CCNG2* signature is found to be an independent predictor of survival that adds new prognostic information to established clinical predictors such as size and age (Supplementary Table 4). **b**, Gene set enrichment analysis (GSEA) for association between high or low *SHARP1* and *CCNG2* expression values and gene sets denoting the activation of specific signalling pathways. The enrichment of HIF-target genes in tumours with low *SHARP1* and *CCNG2* expression was confirmed using an independent statistical method (Supplementary Table 7). Bold, signatures that reach statistical significance. ES, enrichment score; FDR, false discovery rate. **c**, Co-immunoprecipitation (Co-IP) of endogenous SHARP1 with endogenous HIF-1α, from extracts of MDA-231 and Hs578T cells. Asterisk, a background band resulting from the cross-reaction of immunoglobulins (IgGs). In, input. **d**, Quantitative polymerase chain reaction (qPCR) analyses of selected HIF targets in MDA-231 cells stably overexpressing empty vector (Control) or SHARP1, and incubated in low (1%) or normal (20%) oxygen levels. Expression levels are relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), data are normalized to lane 1 (for analyses of additional HIF targets, see Supplementary Fig. 6; and for control of SHARP1 overexpression, see Fig. 3a). The mean and s.d. of one representative experiment, out of three independent experiments performed in triplicate, are shown. **e**, qPCR analyses of selected HIF targets in MII cells transfected with the indicated siRNAs (Supplementary Table 10), and incubated at the indicated oxygen levels (for analyses of additional HIF targets, see Supplementary Fig. 7). The mean and s.d. of one representative experiment, out of three independent experiments performed in triplicate, are shown. **f**, Immunohistochemistry was used to stain TNBC samples for SHARP1 and the HIF target CAIX ($n = 62$). Left, error bars represent mean and s.e.m. The *P* value was calculated using the Student's *t*-test. Right, representative immunohistochemistry. Scale bars, 100 μm.

activation of HIF targets (data not shown), prompting us to focus on SHARP1 for further analyses.

SHARP1 levels seem elevated in the TNBC non-metastatic MCF10Atk1 cells (also known as MII cells) compared to more aggressive MDA-231cells (Supplementary Fig. 5). Taking advantage of this differential SHARP1 expression, we reasoned that if SHARP1 opposes HIF activity, gain of SHARP1 in MDA-231 cells should blunt HIF transcriptional responses, and conversely, loss of SHARP1 in MII cells should enhance HIF-dependent responses. HIF-1α and HIF-2α regulation occurs in response to microenvironmental oxygen levels[7]. Under hypoxia, HIF levels are stabilized to activate a key set of target genes, such as angiopoietin-like 4 (*ANGPTL4*), *LOXL2* and *miR-210,* that have been involved in multiple steps of the metastatic cascade of oestrogen -negative breast cancer cells[13–15]. As expected, these genes were upregulated by hypoxia in control MDA-231 cells, but gain of SHARP1 dampened these inductions (Fig. 1d and Supplementary Fig. 6). Similarly, inductions of HIF targets related to tumour metabolism, such as carbonic anhydrase IX (*CAIX*; also known as *CA9*), hexokinase 2 (*HK2*) and pyruvate dehydrogenase kinase 1 (*PDK1*), or the autophagy and stress regulators *BNIP3* and *NDRG1* (ref. 7), were also inhibited by SHARP1 (Fig. 1d and Supplementary Fig. 6). Thus, sustaining SHARP1 expression is sufficient to oppose HIF responses. Conversely, in MII cells, loss of SHARP1 strongly cooperated with a pulse of hypoxia to upregulate the HIF targets vascular endothelial growth factor A (*VEGFA*), *CAIX*, *ANGPTL4*, *HK2* and *PDK1* (Fig. 1e and Supplementary Fig. 7). This indicates that SHARP1 is required to limit HIF activity and is an endogenous buffer against the effects of hypoxia.

To investigate in an unbiased way and at a genome-wide level whether SHARP1 and HIFs control a significantly overlapping set of genes, transcriptomic profiles were obtained from cells stably expressing either short hairpin green fluorescent protein (shGFP) or shRNAs against HIF-1α and HIF-2α, and from cells overexpressing SHARP1. We identified two independent lists of genes differentially expressed after SHARP1 overexpression or after depletion of HIFs (Supplementary Tables 8 and 9, and Supplementary Fig. 8). When these lists were compared, we found a highly statistically significant overlap between the two lists (Fischer's test, $P < 10^{-73}$), supporting the idea that SHARP1 is a global inhibitor of HIF-1α and HIF-2α gene responses. From these microarrays, we generated a list of genes repressed by SHARP1 (Supplementary Table 6) and found that this signature has prognostic value in TNBC data sets. Tumours expressing high levels of such genes (that is, low SHARP1 activity signature) (Supplementary Fig. 9) display increased propensity to distant metastasis than tumours expressing low levels of the same signature (that is, those retaining high SHARP1 activity). Cox multivariate analyses revealed that the signature of SHARP1-repressed genes did not add prognostic information when combined to a signature of high HIFs activity ($P = 0.4434$); a similar result was obtained from the combination of the *SHARP1* and *CCNG2* signature with HIF activity signature ($P = 0.3072$), indicating that the prognostic value of SHARP1 activity is contained in the prognostic value of HIFs.

These results prompted us to verify the inverse correlation between HIF activity and *SHARP1* expression in primary human TNBC. Levels of *VEGF* transcripts and of other HIF targets were found to decline in tumours displaying increasing levels of *SHARP1* (Supplementary Fig. 10). Notably, immunohistochemical analysis of an independent cohort of 62 TNBC diagnosed at our institution showed an inverse relationship between SHARP1 and CAIX, a validated immunohistological marker of HIF activity in invasive breast cancer[16] (Fig. 1f).

We then investigated the functional relevance of the SHARP1–HIF axis for malignant behaviour of TNBC cells. *In vivo*, both the depletion of HIFs and gain of SHARP1 opposed lung colonization of MDA-231 cells after tail-vein injection in recipient mice (Fig. 2a). SHARP1-mediated inhibition was partially rescued by overexpression of PA-HIF-1α, a constitutively active and pVHL-insensitive HIF-1α[7]

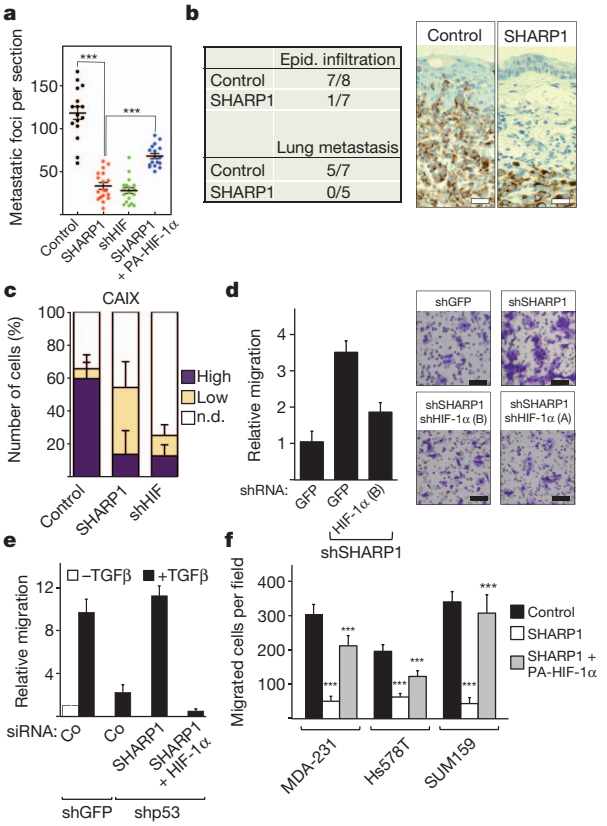

Figure 2 | SHARP1 is a suppressor of invasion, migration and metastasis by inhibiting HIFs. **a**, Lung colonization assay of mice intravenously injected with MDA-231 cells stably transduced with the indicated expression vectors or shHIFs. The control is an empty vector (pLPCX). The plot shows the number of metastatic foci per section, error bars represent mean and s.e.m. (two sections per lung, $n > 8$ mice; $^{***}P < 0.0001$, based on Student's t-test). **b**, Quantification of lung colonization and local invasiveness of primary tumours emerging from orthotopically injected control and SHARP1-overexpressing MDA-231 cells (left panel), and representative images (right panel). Tumours were stained for human cytokeratin. Scale bars, 50 μm. Epid, epidermal. **c**, The HIF-target CAIX protein level was analysed by fluorescent immunohistochemistry in tumours ($n = 7$) emerging after fat pad injection of Control and SHARP1-expressing MDA-231 in mice. Depletion of HIF-1α and HIF-2α served as a positive control for CAIX inhibition (see Supplementary Fig. 11 for comparable results with Glut1 and representative images). n.d., signal not detectable. The error bars represent mean and s.e.m. **d**, Trans-well migration assay of MII cells, a non-metastatic TNBC model system, stably expressing the indicated shRNAs (see Supplementary Table 10). The plot shows the quantification of the area covered by the migrated cells, relative to the first lane that was set to 1. A similar result was obtained with an independent shRNA for HIF-1α (data not shown; see Supplementary Table 10, sequence A). A, B, two sequences targeting HIF-1α (these are given in Supplementary Table 10) Right panels, representative images of the filters (see Supplementary Fig. 14 for knockdown controls). Scale bars, 50 μm. The mean and s.d. of one representative experiment, out of three independent experiments performed in triplicate, are shown. **e**, Trans-well migration assay in MDA-231 cells. Cells expressing shRNA targeting GFP or an shRNA targeting mutant p53 were transiently transfected with the indicated siRNAs (siSHARP1 is sequence B in Supplementary Table 10). TGFβ1 was used to trigger cell migration. Graphs show the quantification of cells that passed through the filter, relative to the first lane that was set to 1. Similar results were obtained by transfecting HIF-1β (Supplementary Fig. 15). The mean and s.d. of one representative experiment, out of five independent experiments performed in triplicate, are shown. **f**, Quantification of wound-healing assays of multiple TNBC cell lines stably transfected with the indicated plasmids (see Supplementary Fig. 16 for representative images). The mean and s.d. of one representative experiment, out of three independent experiments performed in triplicate, are shown ($^{***}P < 0.0001$, Student's t-test; the SHARP1 group was compared to the control group, and the group of SHARP1 with HIF-1α was compared to the SHARP1 group).

(Fig. 2a). When cells were injected orthotopically as a primary tumour in the mammary fat pad, we also noticed a dramatic difference in local invasiveness and distant metastasis between control and SHARP1-expressing tumours (Fig. 2b). In agreement with the role of SHARP1 as a HIF inhibitor, experimental tumours derived from SHARP1-expressing cells showed a reduction of the HIF targets CAIX and Glut1 to levels comparable to tumours emerging from HIF-depleted cells (Fig. 2c and Supplementary Fig. 11). Notably, gain of SHARP1 recapitulates the response of TAp63α, as TAp63α-expressing MDA-231 cells also show a severely impaired metastatic spread in tail-vein assays or after their orthotopic injection into the mammary fat pad (Supplementary Fig. 12). This is consistent with *SHARP1* levels being dependent on *TAp63α* in TNBC cell lines (Supplementary Fig. 13).

The above experiments indicate that by antagonizing HIFs, SHARP1 may control migratory and invasive cell behaviours in tumours. To substantiate further this conclusion, we monitored the relevance of SHARP1 for HIF-dependent cell migration *in vitro*. Loss of SHARP1 leads to increased trans-well migration in MII cells, and this effect was rescued by concomitant depletion of HIF-1α (Fig. 2d). As shown in Fig. 2e, similar results were obtained in MDA-231 cells depleted of mutant p53 (a treatment that relieves the inhibition of endogenous TAp63α activity in these cells, raising SHARP1 levels[3,5,17,18]). To extend the validity of this epistasis, gain of HIFs opposes the anti-migratory effects of gain of SHARP1 in other TNBC cell lines, such as SUM159 and Hs578T. SHARP1-expressing cells were unable to migrate in a wound-healing assay, and this could be rescued by over-expression of PA-HIF-1α or PA-HIF-2α (Fig. 2f and Supplementary Figs 16 and 17). These responses occurred in the absence of any notable effect on cell growth (Supplementary Fig. 18, data not shown).

The data presented so far establish SHARP1 as a physiological inhibitor of HIF function. We next sought to determine the mechanism of this inhibition. HIF regulation occurs mainly at the level of protein stability[7]. Notably, SHARP1 overexpression greatly reduced endogenous HIF-1α protein levels, a result confirmed in three independent TNBC cell lines (MDA-231, SUM159 and Hs578T) (Fig. 3a, b). SHARP1 was equally effective against HIF-2α (Supplementary Fig. 19). Intriguingly, the effect of SHARP1 was independent from oxygen levels, as downregulation of HIF-1α protein occurred highly efficiently in cells cultured in both normoxic and in hypoxic conditions (Fig. 3a). In experimental tumours derived from orthotopically injected MDA-231 cells, SHARP1 also downregulated HIF-1α protein levels to those of cells expressing shHIFs (Fig. 3c and Supplementary Fig. 20).

We then examined whether endogenous SHARP1 is a relevant inhibitor of HIF-1α protein levels. For this, we depleted SHARP1 from MII cells and MDA-231 cells. SHARP1 depletion induced robust HIF-1α stabilization, a finding that was confirmed with independent SHARP1 siRNAs (Fig. 3d, e and Supplementary Fig. 21). In agreement with SHARP1 expression being regulated by TAp63α in TNBC cell lines (Supplementary Fig. 13), effective downregulation of endogenous HIF-1α protein could be observed in MDA-231 cells after transfecting an expression vector for TAp63α, or after knockdown of mutant p53 (Supplementary Fig. 22).

This raised the question of how SHARP1 impinges on HIF protein levels. Although oxygen-dependent or pVHL-mediated degradation of HIF-1α or HIF-2α has received considerable attention[7], it is also clear that HIFs are unstable proteins degraded by the proteasome even under hypoxic conditions[19,20]. However, this aspect of HIF regulation has remained only partially understood. We therefore tested to see whether the proteasome is required for the effects of SHARP1. Treatments of MDA-231 or HEK293T cells with proteasome inhibitors effectively opposed SHARP1-mediated HIF-1α degradation, resulting in accumulation of unmodified and polyubiquitinated HIF-1α isoforms (Fig. 3f and Supplementary Fig. 23). Notably, SHARP1 was able to downregulate the levels of wild-type HIF-1α

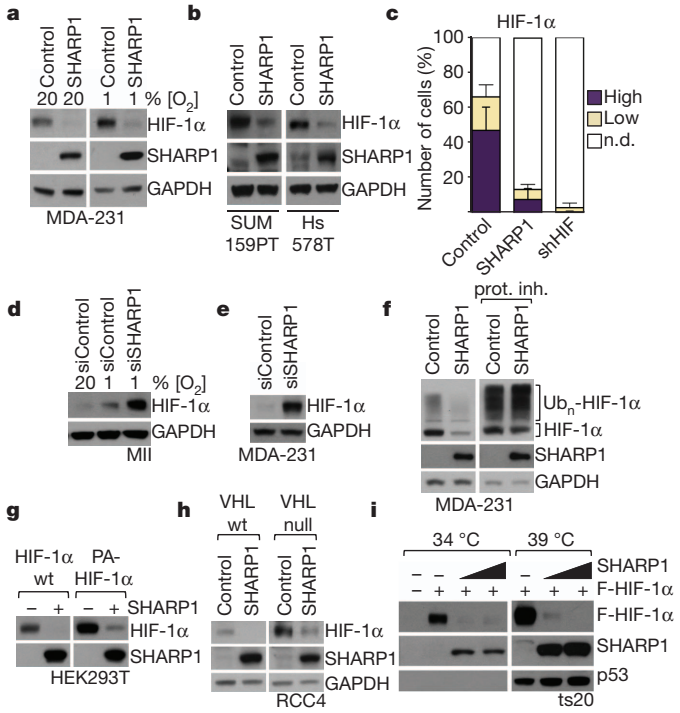

Figure 3 | SHARP1 promotes HIF-1α proteasomal degradation independently of oxygen levels and pVHL. **a**, Western blot analysis of control and SHARP1-overexpressing MDA-231 cells cultured at the indicated oxygen levels for 24 h. **b**, SHARP1 overexpression downregulates HIF-1α protein levels in two additional TNBC cell lines (SUM159 and Hs578T). **c**, Quantification of fluorescent immunohistochemistry of HIF-1α protein in tumours arising from orthotopically injected control and SHARP1-overexpressing MDA-231 cells (n = 7 tumours) (see Supplementary Fig. 20 for representative images). Error bars represent mean and s.e.m. **d**, **e**, Depletion of SHARP1 increases HIF-1α stabilization in MII cells (**d**, after 6 h of hypoxia) and MDA-231 cells (**e**, cultivated in normoxia) (for similar results obtained with an independent SHARP1-siRNA and for control of knockdown, see Supplementary Fig. 21). **f**, Downregulation of HIF-1α protein levels by SHARP1 in MDA-231 cells depends on the proteasome. Prot. Inh., proteasome inhibitors; Ub_n-HIF-1α, polyubiquitinated HIF-1α. **g**, Raising SHARP1 leads to reduction of wild-type (wt) HIF-1α or PA-HIF-1α levels. **h**, Western blot analyses of cell lysates from RCC4 cells, either null for pVHL or pVHL-reconstituted, stably expressing SHARP1 or empty vector as control. **i**, BALB/c 3T3-derived ts20 cells (BALB/c3T3ts20), bearing a temperature-sensitive (ts) ubiquitin conjugating enzyme (E1) mutant, were transfected with Flag–HIF-1α (F-HIF-1α), alone or with increasing doses of SHARP1. Cells were cultured at 34 °C and shifted at 39 °C to inactivate E1. Western blot analysis of p53 ensures the efficient inhibition of ubiquitin-dependent pathways[24]. In case of vertical slicing of blots of the same cell line, samples were obtained from the same experiment and pictures derive from blots processed in parallel.

and of a hydroxylation-mutant, and therefore prolyl hydroxylase-insensitive, PA-HIF-1α (Fig. 3g), and could effectively operate in VHL-mutant renal cell carcinoma (RCC4) cells (Fig. 3h). Thus, SHARP1 curtails HIF-1α activity independently of the pVHL pathway.

To test whether ubiquitination is involved in SHARP1-mediated HIF-1α degradation, we used cells carrying a temperature-sensitive mutant of the E1 ubiquitin-activating enzyme (UBE1). SHARP1 is equally active in permissive (34 °C) and non-permissive (39 °C) conditions (Fig. 3i). As a positive control for this assay, mdm2-mediated degradation of p53 was inhibited in non-permissive conditions (Fig. 3i). This suggests that SHARP1 operates independently of HIF-1α ubiquitination. Consistent with this, overexpression of SHARP1 does not increase polyubiquitination of HIF-1α, which is different from the effects of transfected VHL (Supplementary Fig. 24).

For proteasomal-dependent degradation, we proposed as an alternative mechanism to ubiquitination that SHARP1 may present HIF-1α to the proteasome. The proteasome has previously been shown to bind and degrade specific short-lived proteins in an ubiquitin-independent manner[21,22], matching the effects of SHARP1. Interestingly, unmodified HIF-1α can directly bind the 20S α4 subunit of the proteasome[23]. Through co-immunoprecipitations, both HIF-1α and SHARP1 associate with the 20S proteasomal subunit (Fig. 4a).

To capture in living cells if SHARP1 is instrumental for HIF-1α recognition by the proteasome, we stabilized protein complexes by treating HEK293T and MDA-231 cells with the bifunctional and cell permeable crosslinker DSP (dithiobis(succinimidylpropionate)) before cell lysis and co-immunoprecipitation. Expression of SHARP1 increased the association of HIF-1α with the 20S subunit (Fig. 4b and Supplementary Fig. 25). Given the requirement of SHARP1 for HIF-1α instability, we then tested the relevance of endogenous SHARP1 for the formation of the HIF-1α–20S complex. In co-immunoprecipitation assays, HIF-1α failed to efficiently associate to the 20S subunit in lysates of SHARP1-depleted cells, indicating that SHARP1 is essential for efficient recognition of HIF-1α by the 20S proteasome (Fig. 4c).

We next questioned whether the capacity to bind HIF and the proteasome is instrumental for SHARP1 function. To address this question we first dissected the structural requirements of SHARP1 to dock HIF-1α to the 20S by comparing different SHARP1 deletion constructs using co-immunoprecipitation assays. The amino-terminal basic helix–loop–helix (bHLH) domain of SHARP1 (Fig. 4d) was

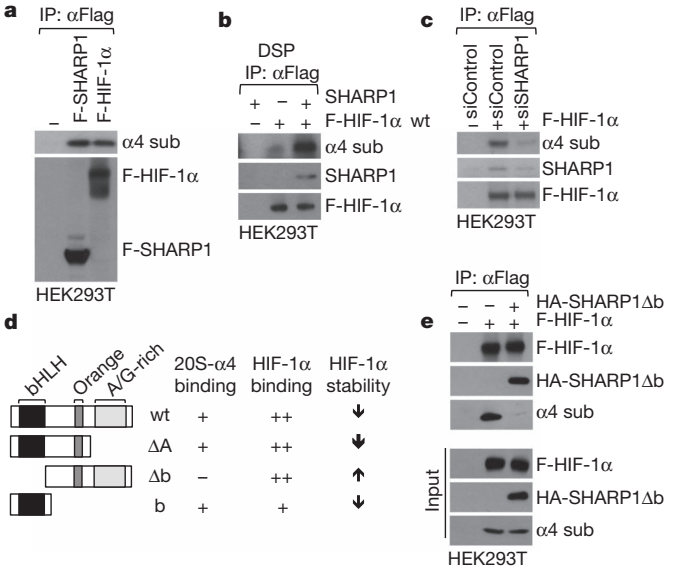

Figure 4 | SHARP1 promotes the interaction of HIF-1α with the proteasome. **a**, Co-immunoprecipitation of the α4 subunit of the 20S proteasome with overexpressed SHARP1 and HIF-1α. **b**, HEK293T cells expressing Flag-tagged HIF-1α alone or together with untagged SHARP1, were incubated with the cell-permeable DSP crosslinker before collection. Extracts were subjected to anti-Flag immunoprecipitation (IP), and co-precipitated endogenous α4 subunit of the 20S proteasome was detected by immunoblotting after de-crosslinking of the lysate. **c**, HEK293T cells were transfected with the indicated siRNAs and with Flag-tagged HIF-1α. After collection, extracts were subjected to anti-Flag immunoprecipitation; the co-precipitating endogenous 20S α4 proteasome subunit and endogenous SHARP1 proteins were detected by immunoblotting. **d**, Diagrams of the domains of SHARP1 and corresponding deletion mutants (ΔA, Δb and b) used in co-immunoprecipitation experiments with HIF-1α and the α4 subunit of the 20S proteasome, and a summary of the results (experimental data are shown in Supplementary Figs 26 and 27). Plus and minus symbols, presence or absence of binding of the different SHARP1 mutants with the 20S α4 subunits or HIF-1α. Arrows, effect of the SHARP1 mutant (increasing or decreasing HIF-1α stability). **e**, A SHARP1 deletion lacking its bHLH domain inhibits the binding of HIF-1α to the α4 proteasome subunit.

required and sufficient for proteasomal association, whereas for HIF association, the bHLH is sufficient but not essential (Fig. 4d and Supplementary Figs 26 and 27). The sole bHLH domain is capable of triggering HIF-1α and HIF-2α instability in a proteasome-dependent manner, thus recapitulating the effects of full-length SHARP1 (Fig. 4d and Supplementary Figs 28 and 29). Interestingly, a bHLH-deleted version of SHARP1 (SHARP1-Δb), retained HIF-1α association but lost proteasomal recognition (Fig. 4d and Supplementary Figs 26 and 27). Accordingly, expression of SHARP1-Δb led to HIF stabilization (Supplementary Figs 28 and 29) by preventing the binding of HIF-1α to the proteasome (Fig. 4e, as assayed by anti-HIF-1α co-immunoprecipitation). We conclude from these results that SHARP1 needs to associate with both HIFs and the proteasome to cause HIF degradation.

In the present work, we have presented a mechanistic link between SHARP1 and HIF activities, and we have provided clinical and functional evidence suggesting that this pathway is exploited in aggressive TNBC. A notable result was the identification of SHARP1 as an essential cellular determinant of the intrinsic instability of HIF proteins. SHARP1 acts in both normoxic and hypoxic cells, and irrespective of pVHL or ubiquitination pathways. We propose that, acting in parallel to oxygen-dependent mechanisms for HIFs degradation, elevated SHARP1 levels curtail the effects of hypoxic as well as oncogenic HIF stabilization in breast cancer. HIF activation represents a final common event in cancer pathogenesis in a variety of tumours; as such, the identification of SHARP1 uncovers the possibility of manipulating HIF-induced tumour progression.

METHODS SUMMARY

For trans-well migration assays, MDA-MB-231 cells were transfected with the indicated siRNAs, starved for 48 h in medium without serum and then plated on polyester (PET) inserts. The medium in the top and bottom chambers was supplemented with the TGFβ receptor inhibitor SB505124 (10 μM) or TGFβ1 (5 ng ml^{-1}) to induce cell migration. For protein–protein interactions in live cells between HIF-1α and the 20S α4 subunit, cells were treated with the cell-permeable crosslinker DSP (dithiobis-(succinimidyl propionate)) (2.5 mM) before harvesting. For ubiquitination assays with 3T3-ts20 cells, these cells were incubated at 39 °C for 8–12 h, transfected and then cultured at 39 °C for an additional 48 h. For all hypoxic treatments, cells were kept in a hypoxic cabinet and cultured at 1% oxygen level for the indicated times (for a complete description of methods and protocols, please see the Supplementary Information).

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