Differential regulation of the REGγ–proteasome pathway by p53/TGF-β signalling and mutant p53 in cancer cells

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Proteasome activity is frequently enhanced in cancer to accelerate metastasis and tumorigenesis. REGγ, a proteasome activator known to promote p53/p21/p16 degradation, is often overexpressed in cancer cells. Here we show that p53/TGF-β signalling inhibits the REGγ–20S proteasome pathway by repressing REGγ expression. Smad3 and p53 interact on the REGγ promoter via the p53RE/SBE region. Conversely, mutant p53 binds to the REGγ promoter and recruits p300. Importantly, mutant p53 prevents Smad3/N-CoR complex formation on the REGγ promoter, which enhances the activity of the REGγ–20S proteasome pathway and contributes to mutant p53 gain of function. Depletion of REGγ alters the cellular response to p53/TGF-β signalling in drug resistance, proliferation, cell cycle progression and proteasome activity. Moreover, p53 mutations show a positive correlation with REGγ expression in cancer samples. These findings suggest that targeting REGγ–20S proteasome for cancer therapy may be applicable to human tumours with abnormal p53/Smad protein status. Furthermore, this study demonstrates a link between p53/TGF-β signalling and the REGγ–20S proteasome pathway, and provides insight into the REGγ/p53 feedback loop.
REGγ (also known as PA28γ, PSME3 or Ki antigen) belongs to the REG or 11S family of proteasome activator that has been shown to bind and activate 20S proteasomes. REGγ activates the ubiquitin-independent degradation of steroid receptor coactivator-3 (ref. 3). In addition, REGγ also promotes degradation of several important regulatory proteins, including the cyclin-dependent kinase inhibitor p21 (refs 4,5). Moreover, REGγ enhances the MDM2-mediated ubiquitination and proteasomal degradation of tumour suppressor p53, inhibiting p53 accumulation and apoptosis after DNA damage. Previous reports showed that REGγ-knockout mice and cells displayed reduced growth, decreased cell proliferation and increased apoptosis. Growing evidence suggests that REGγ is involved in cancer progression. REGγ was reported to be overexpressed in the breast, thyroid, colorectal, lung and liver cancers. However, the molecular mechanisms by which REGγ is overexpressed in multiple cancer tissues and cell lines largely remains unknown.

TP53 is a sequence-specific transcription factor, which is present in a very low amount in normal cells. In response to various types of genotoxic stress, p53 is activated to regulate the expression of multiple genes. The regulation of p53-responsive genes produces proteins that interact with numerous other cellular signalling pathways, and a number of positive and negative autoregulatory feedback loops are generated. The biological implications of these loops mainly depend on the function of the transcriptional targets. Yet, the p53 transcription targets and its feedback loops are not fully understood.

Transforming growth factor-β (TGF-β) is a ubiquitously expressed pleiotropic cytokine that has important roles in cellular function such as apoptosis, cell cycle arrest, homeostasis, immune regulation and angiogenesis. TGF-β is a potent activator of cytosstatic programme in epithelial cells. In the classical TGF-β pathway, ligand binding induces the assembly of type I and type II serine/threonine kinase receptors and subsequent phosphorylation of the type I receptor by constitutively active type II receptors. The activated type I receptor phosphorylates cytoplasmic proteins called Smads, thus allowing the formation of heteromeric Smad complexes and their subsequent translocation to the nucleus. Once in the nucleus, these complexes control gene expression through interaction with transcription factors, co-activators and co-repressors. Although TGF-β is considered a double-edged sword for its tumour suppressive and tumour-promoting functions, genetic loss of Smad function through deletion, mutation and subsequent loss of heterozygosity is a frequent event in tumours. It is noteworthy that p53 is known to be required for full activity of TGF-β-mediated regulation by cooperating with Smads. Inactivation of p53 has been linked to alteration of Smad-dependent TGF-β signalling.

Mutation of the p53 tumour suppressor gene is one of the most frequent genetic alterations in human tumours and poses a critical event in tumorigenesis, affecting tumour development, progression and responsiveness to therapy. Approximately 50% of human cancers have p53 loss-of-function mutations. Mutant p53 knockin mice showed a higher frequency of tumour development and increased metastatic potential compared with p53-deficient mice. Tumour-associated forms of mutant p53 can contribute to genomic instability by abrogating the mitotic spindle check point and, consequently, facilitating the generation of aneuploid cells. To date, three molecular mechanisms have been described for gain of function (GOF) of mutant p53: (1) mutant p53 can bind to and inactivate the tumour suppressor proteins such as p63 and p73; (2) mutant p53 can bind to DNA and control the transcriptional regulation of putative target genes; and (3) mutant p53 can take part in the formation of large transcriptional competent complexes through which the expression of its target genes is regulated.

Here we report how the REGγ–20S proteasome pathway is enhanced during cancer progression. The recruitment of Smad3 and p53 at the intercalated p53RE/Smad-binding element (SBE) region in response to TGF-β provides a convergent action on REGγ expression by these tumour suppressive pathways. In addition, REGγ overexpression in diverse cancer cell lines can be specifically driven by mutant p53 that is recruited to upstream of the REGγ promoter. Moreover, mutant p53 attenuates binding of TGF-β-activated Smad3/4 complex and Nuclear receptor corepressor (N-CoR) to the SBE region of the REGγ promoter, highlighting its novel GOF ability. Silencing of REGγ alters cellular response to p53 and TGF-β signalling in drug resistance, cell proliferation, 20S proteasome activity and cell cycle progression. Our results demonstrate a role for p53/TGF-β signalling in the regulation of the REGγ–20S proteasome pathway, a new GOF for mutant p53 in enhancing the REGγ–20S proteasome pathway in cancer cells and further insight into the p53/REGγ feedback loop.

### Results

**TP53 represses REGγ via p53RE to generate a feedback loop.** In an effort to analyse transcriptional regulation of REGγ, we cloned ~2.5 kb genomic DNA sequences upstream of the REGγ translational initiation codon. To define the transcriptional initiation site, we synthesized appropriate primers for REGγ and performed 5'-RACE (rapid amplification of complementary DNA ends) as described previously. The result indicated that the REGγ transcript had a 5'-end (defined as +1) within 130 bp from the ATG site (Supplementary Fig. S1a). When fused to a luciferase reporter, the 2.5-Kb (−2,470/+130) and the 1.3-Kb (−1,177/+130) untranslated region (UTR) of REGγ had similar luciferase activity, whereas the (−2,470−1,177) region produced no activity, indicating that the 1.3-Kb (−1,177+/130) UTR contains the key regulatory components for transcriptional regulation of REGγ (Supplementary Fig. S1b). Bioinformatic analysis, using the NCBI database, revealed that the human REGγ UTR contains four putative p53 DNA-binding sites (Fig. 1a). A series of deletion constructs of REGγ-luc reporters were generated (Supplementary Fig. S1c) for measuring transcriptional activity. The derepression in the (−518) construct suggested a potential negative regulatory region flanking the (−738/−518) region, which correlates with the location of p53RE-2 and p53RE-3 (Supplementary Fig. S1d).

To investigate whether p53 transcriptionally regulates REGγ, we co-transfected the REGγ-luc reporter into H1299 cells. Repression was observed when the reporter construct was co-transfected with different doses of p53 (Fig. 1b). Derepression of REGγ also occurred in p53-depleted HCT116 cells (Supplementary Fig. S2a). In response to Nutlin-3, inhibition of the REGγ transcript was detected in HCT116 (p53+/+ ) but not in the isogenic HCT116 (p53−/−) cells (Fig. 1c and Supplementary Fig. S2b). Silencing p53 by short interfering RNA (siRNA) in multiple cancer cells greatly increased REGγ mRNA levels compared with controls (Fig. 1d and Supplementary Fig. S2a). Similar to Nutlin-3, cisplatin and etoposide (ETO) also significantly reduced both REGγ mRNA and protein expressions (Fig. 1e,f). Likewise, upregulation of REGγ was also observed in p53−/− mouse embryonic fibroblast cells (Fig. 1g).

Next, we attempted to identify the regulatory region conferring p53 responsiveness within the REGγ promoter. Interestingly, only p53RE-3 displayed strong binding to p53 by electrophoretic mobility shift assay (EMSA; Supplementary Fig. S2d). Mutation of the p53RE-3 construct abolished its response to p53 inhibition, validating that p53 binds to p53RE-3 to repress REGγ transcription (Fig. 1h). We further confirmed p53 binding to p53RE-3 by
Figure 1 | (a) Schematic representation of putative p53-responsive elements (p53REs) with 1.3 kb region of the REGy promoter. Dark grey colour represents critical p53RE-3. (b) H1299 cells were co-transfected with REGy reporter construct along with an empty vector or increasing amounts of p53 for 24 h before lysis and were analysed for luciferase activity. The average was calculated based on three independent experiments. Error bars show the mean ± s.d. from three technical replicates (two-tailed Student’s t-test, *P<0.05). (c) HCT116 p53 (+/+) and HCT116 p53 (−/−) were treated with 10 μmol l⁻¹ Nutlin-3 for indicated time points to perform quantitative RT–PCR analysis. The average was calculated based on three independent experiments. Data are representative of three technical repeats with mean ± s.d. (two-tailed Student’s t-test, *P<0.05, **P<0.005). (d) AS49, HepG2 and MCF-7 cells were transfected independently with siRNA specific for p53 (20 nM for 48 h) and total RNA was isolated. Data represent average of three independent experiments. Data show the mean ± s.d. from three technical replicates (two-tailed Student’s t-test, *P<0.05). (e,f) AS49 cells were treated with different anticancer drugs such as Nutlin-3 (10 μmol l⁻¹), Cisplatin (5 μg ml⁻¹), ETO (10 μmol) and Adriamycin (1 μM), and were analysed by (e) RT–PCR and by (f) western blotting. (e) Error bars show the mean ± s.d. from three technical replicates. (Two-tailed Student’s t-test, *P<0.05, **P<0.005). (g) Comparative analysis of REGy mRNA and protein levels in mouse embryonic fibroblast (MEF) p53 (+/+) and MEF p53 (−/−) cells. (h) H1299 cells were co-transfected with wild-type (2 μg) or mutated p53RE (2 μg) REGy luciferase reporter constructs along with the p53 plasmid (75 ng) for 24 h and then analysed for luciferase activity. Data are representative of three independent experiments. Error bars show the mean ± s.d. from three technical replicates. (Two-tailed Student’s t-test, *P<0.05, **P<0.005). (i) A549 cells (upper panel) and MEF cells (lower panel) were treated with Nutlin-3 for 24 h, and EMSA assays were performed with the double-stranded oligonucleotides containing the p53RE from the REGy promoter. (j) Schematic representation of ChIP primers. A549 cells (upper two panels) and MEF cells (lower panel) were independently treated with Nutlin-3a for 24 h, and ChIP assays were performed with anti-p53 antibody. (k) ChIP analysis of REGy promoter in A549 cells at indicated time periods after Nutlin-3 (10 μmol l⁻¹) treatment.

EMSA assay, which resulted in the formation of a p53–DNA complex (Fig. 1i, upper panel). We obtained similar results for the p53RE-3 probe using recombinant p53 protein (Supplementary Fig. S2e). Intriguingly, EMSA analysis also revealed p53 binding to a probe derived from mouse REGy UTR (Fig. 1i, lower panel).

To determine whether p53 directly binds to p53RE in vivo, a chromatin immunoprecipitation (ChIP) assay was performed using the primers derived from the REGy promoter (Fig. 1j). The anti-p53 antibody specifically pulled down DNA fragments corresponding to the p53RE-3 region (Fig. 1j, upper two panels and Supplementary Fig. S2f). Moreover, the ChIP assay also suggested p53 binding to p53RE in the UTR of mouse REGy using mouse embryonic fibroblast cells treated with Nutlin-3 (Fig. 1j, lower panel). It was previously reported that p53 has been
associated with the recruitment of histone deacetylase to repress its target genes. Thus, the ChIP assays also demonstrated a time-course-dependent recruitment of p53 and mSin3A-HDAC1 co-repressor complex to the REG promoter (Fig. 1k). Importantly, this co-repressor complex was preferentially associated with a repressive chromatin status in the REG promoter, as indicated from the enrichment of Me-H3K9 (Fig. 1k), a marker of repressed chromatin. Taken together, these data demonstrate that REG negatively regulates p53 through a feedback loop, as loss of p53 and Smad3/4 act as transcriptional co-repressors on the REG promoter.

Next, we aimed to address the molecular details of the potential interaction of p53 and Smad proteins at the p53RE-3/SBE-4 region. We silenced Smad3 expression in the A549 cell line to determine whether recruitment of p53 to REG promoter depends on Smad3 in response to TGF-β. ChIP analysis showed no recruitment of Smad3, p53 and N-CoR in Smad3-depleted cells in the presence of TGF-β, which was comparable with control cell lines, suggesting that activated Smad3 interacts with p53 at the p53RE-3/SBE-4 region in response to TGF-β, and entails recruitment of p53 to the REG promoter (Fig. 3i). To define binding of these proteins to p53RE-3/SBE-4 regulatory region, we performed an EMSA assay. Incubation of nuclear extracts with a 26-nt probe resulted in formation of a putative p53–Smad–DNA complex, which is enhanced in the presence of TGF-β (Fig. 3g, lanes 2 and 3). Addition of both antibodies against p53 and Smad3 nearly abolished all bands (Fig. 3g, lane 6), indicating that these complexes contained both p53 and Smad3 proteins. Consistent with our in vitro protein–DNA interaction analysis, ChIP assays revealed that TGF-β stimulated coregulation of Smad3/p53/N-CoR to this region, suggesting the cooperation between p53 and Smads to further repress REG expression (Fig. 3h). Intriguingly, sequence analysis of the mouse REG promoter also showed p53RE/SBE site between the (−2,939/−2,912) region (Fig. 3i). ChIP assay disclosed binding of Smad3/p53/N-CoR to this region as well (Fig. 3i). Next, we depleted expression of N-CoR using specific siRNA to validate the biological impact of N-CoR on REG regulation. Reverse transcriptase–PCR (RT–PCR) analysis revealed that the REG mRNA level was elevated in N-CoR knockdown cells (Fig. 3j). Taken together, our data suggest that synergism/collaboration between p53 and TGF-β occurs on the REG promoter through the p53RE/SBE region, which contributes to further inhibition of the REG–proteasome activity. Importantly, our data suggest crosstalk between these two key pathways, not only in human cancer cell lines but also in non-cancer mouse cells.

Mutant p53 interacts and recruits p300 to induce REG. Mutant p53 was previously detected on the promoters of some target genes, including CD95 (ref. 50), EGR1 (ref. 51), MSP-1 (ref. 52), GRO-1 (ref. 53), ID2 (ref. 54) and ID4 (ref. 55), and this was observed in the absence of external stimuli, implying that mutant p53 can directly bind to and regulate its target genes. To understand whether mutant p53 regulates REG, we co-transfected the REG–luc reporter with increasing amounts of mutant p53–R175H and observed the dose-dependent transactivation of the REG–luciferase reporter (Fig. 4a). Consistently, expressing
REGγ reporter construct in UMSCC-1 cell lines stably expressing an empty vector, p53 or p53-R175H also suggested a role for mutant p53 to promote REGγ transcription (Supplementary Fig. S5a). Moreover, all coexpressed constructs containing hotspot p53 mutations significantly activated transcription of REGγ reporter in the H1299 cell (Fig. 4b). To investigate the impact of endogenous mutant p53 on REGγ expression, we silenced mutant p53 in multiple cancer cells. Depletion of mutant p53 in these cells reduced the level of REGγ transcription of REGγ (Fig. 4c–d, and Supplementary Fig. S5b–c).
Conversely, H1299 cells stably expressing p53-R175H strongly induced REGγ protein levels compared with control cells (Fig. 4e, left panel). Strikingly, immortalized oral cancer cells from mice with p53 mutation at amino acids 172 (R172H) displayed significant upregulation in REGγ protein level compared with p53−/− oral cancer cells (Fig. 4e, right panel).

Next, reporter assays were performed with various truncated REGγ-luc constructs to determine the region responsive to
mutant p53. The 1.39Kb REGγ-luc reporter construct (−1,177/−738) was responsive to mutant p53 transactivation, whereas a truncated REGγ-luc construct (−738/+130) remained unresponsive to mutant p53 (Supplementary Fig. S5d), indicating a cis-element within the (−1,177/−738) region for mutant p53. To further define the mutant p53-responsive region in the REGγ UTR, we generated different REGγ-Luc deletion constructs (Fig. 4f) and found that the region between (−1,071/−969) is essential for mutant p53-dependent transcription (Fig. 4g). Deletion of this region (−1,071/−969) abolished the capability of mutant p53 to transactivate the REGγ promoter (Fig. 4h), indicating that sequence from (−1,071/−969) may be required for mutant p53 binding. ChIP assays were carried out to examine the association of endogenous mutant p53 with this regulatory region. Following immunoprecipitation of chromatin from H1299 p53-R175H stable cell lines transfected with either siRNA control or siRNA, we observed recruitment of mutant p53 to a region flanking (−1,144/−752) in the REGγ UTR, but not in regions 2 kb further upstream or in cells having depleted mutant p53 (Fig. 4i). We also examined REGγ promoter occupancy by naturally occurring mutant p53 with ChIP analysis in human breast cancer and colon cancers cell lines. We detected
recruitment of mutant p53 (p53-R280K and p53-R273H) to REGγ promoters in MDA-MB-231 and ARO cells (Fig. 4j, upper two panels). Interestingly, ChIP analysis also revealed that p300 bound to the REGγ promoter in mutant p53 (MDA-MB-231 and ARO)-containing cells but not to cells with depleted mutant p53 (Fig. 4j, lower two panels). Altogether, our results demonstrate that the REGγ–20S proteasome pathway is likely to be a common target for different mutant p53 proteins in multiple human cancer cells and mouse oral cancer cells. Moreover, induction of REGγ occurs through recruitment of mutant p53 proteins along with p300 onto the specific regulatory region in REGγ UTR. These findings disclose a new regulatory venue for mutant p53-mediated expression of proteasome activator REGγ in different cancer cell types.

**Mutant p53 prevents Smad3/N-CoR formation on REGγ promoter.** A previous study showed that mutant p53 attenuates the TGF-β pathway by repressing the TGF-βRII gene, delaying or reducing phosphorylation of Smad2 by TGF-βRI[57]. Because of the negative and positive responses of REGγ promoter to TGF-β and mutant p53, respectively, we investigated the effect of TGF-β/Smad3/4 signalling on REGγ expression in mutant p53-expressing cells. We transfected p53-null H1299 cells with Smad3/4 and p53-R175H expression plasmids independently or in combination, and found that mutant p53 was able to attenuate the Smad3/4-mediated inhibition of REGγ promoter in fold changes (Fig. 5a). Furthermore, we also observed a significant reduction in REGγ mRNA and protein levels in H1299 control cells when stimulated with TGF-β, whereas this TGF-β-mediated inhibition was alleviated in cells harbouring p53-R175H (Fig. 5b,c). We obtained similar results in naturally harbouring mutant p53 MDA-MB-231 (p53-R280K) and MDA-MB-1386 (p53-R282W) cells by comparing cells with or without mutant p53 silencing (Fig. 5d,e). These results prompted us to test the influence of mutant p53 protein on binding of TGF-β-activated Smad3/4 complex to the REGγ promoter. For comparison, H1299 cells were infected with a control viral vector, or vectors encoding p53 or p53-R175H, followed by ChIP analysis. We detected the recruitment of Smad3 to REGγ promoter in the presence of TGF-β in control and p53-expressing cells (Fig. 5f). Conversely, we observed little binding of Smad3 in p53-R175H-expressing H1299 cells (Fig. 5f, upper panel). We also validated these observations by ChIP assays in UMSCC-1 cells, stably integrated with an empty vector, p53 or hotspot p53-R175H mutant allele (Fig. 5f, lower panel). We obtained similar results in naturally occurring mutant p53 MDA-MB-231 (Fig. 5g) and stably expressing mutant p53 H1299 cell lines (Fig. 5h), respectively.

In addition, we examined the effect of mutant p53 on Smad3/4-binding affinity to corresponding SBE-4 on the REGγ promoter. Nuclear extracts were prepared from H1299 cells co-transfected with Smad3/4, p53 and p53-R175H expression plasmids, alone or in combination (Smad3/4 + p53 or Smad3/4 + p53-R175H), followed by TGF-β treatment. We believe the EMSA assay probe containing SBE-4 displayed formation of a Smad3/4–DNA complex, as this complex was strongly enhanced after TGF-β stimulation and attenuated after the addition of Smad antibodies (Fig. 5i, lanes 3 and 10). Strikingly, the binding capacity of this Smad3/4 complex to the corresponding SBE-4 oligo was completely abolished by mutant p53-R175H (Fig. 5i, lane 9), suggesting that mutant p53 proteins antagonize TGF-β signalling by inhibiting the binding of Smad complexes to regulatory elements on REGγ promoter. Moreover, we detected a TGF-β time-course-dependent increase of Smad3/N-CoR occupancy at the REGγ promoter in control cells. Conversely, binding of Smad3/N-CoR to the REGγ promoter sharply decreased in p53-R175H-expressing cells. Interestingly, mutant p53 recruited coactivator p300 with increasing strength to the REGγ promoter with TGF-β treatment (Fig. 5j). We also obtained similar results in MDA-MB-231 breast cancer cells (Fig. 5k). Consistent with previous report that mutant p53 attenuates the TGF-β pathway, we also found reduced phosphorylation of R-smad (Supplementary Fig. S6a). Taken together, these data demonstrate that mutant p53, but not p53, is capable of interrupting TGF-β-induced recruitment of Smad complexes to the REGγ promoter by attenuating the DNA-binding capacity of Smad3/4 complex to the corresponding SBE. Furthermore, the switch between the recruitment of N-CoR, which promotes histone deacetylation, and that of p300, which should result in increased histone acetylation, provides additional explanation for the transcriptional activation of REGγ in mutant p53 harbouring cells in response to TGF-β.

**REGγ alters cellular response to p53 and TGF-β signalling.** Next, we aimed to address the impact of REGγ regulation on p53, TGF-β and mutant p53 cellular activities. We treated the A549 cell lines with anticancer drugs and performed cell proliferation assay. We observed that REGγ knockdown cell lines showed less resistance to ETO and Adriamycin treatment as compared with control cells (Fig. 7a and Supplementary Fig. S8). Thus, depletion of REGγ sensitizes cells to genotoxic insults. Furthermore, we measured apoptotic levels by poly (ADP-ribose) polymerase cleavage (an apoptotic marker). We detected significantly more apoptosis in A549 cells than in ARO cells with stable REGγ knockdown (Fig. 7b). Next, we treated A549 cell lines with TGF-β and observed that REGγ-depleted cells were more sensitive to TGF-β treatment and were less proliferative in comparison with control cells (Fig. 7c).

Moreover, overexpression of REGγ attenuated the effect of ETO and TGF-β on cell cycle progression, and enhanced S and G2/M phase to accelerate DNA synthesis (Fig. 7d,e and...
Figure 5 | Mutant p53 antagonizes recruitment of the Smad3/N-CoR complex on the REGγ promoter. (a) H1299 cells were co-transfected with Smad3/4 and p53-R175H expression plasmids independently or in combination and were analysed for luciferase activity. Data are representative of three technical repeats with mean ± s.d. (two-tailed Student’s t-test, **P<0.005). (b, c) H1299 cells stably expressing either p53-R175H mutant or empty vectors (E.V) were left untreated or treated with 5 ng ml⁻¹ TGF-β and were analysed by RT–PCR and western blotting, respectively. Data are representative of three technical repeats with mean ± s.d. (two-tailed Student’s t-test, **P<0.005, ***P<0.0005). (d, e) MDA-MB-231 and MDA-MB-1386 cells were left non-transfected (−) or transfected (+) with siRNA (20 nM) against mutant p53, and then stimulated with TGF-β and analysed by quantitative RT–PCR. Data are representative of three technical repeats with mean ± s.d. (two-tailed Student’s t-test, **P<0.005, ***P<0.0005). (f) H1299 cells were infected either with an E.V, p53 or p53-R175H encoding vectors, and left untreated or treated with 5 ng ml⁻¹ TGF-β for 24 h (upper panel). UMSCC-1 cells stably expressing an empty vector, p53 or p53-R175H vectors were treated similar to those in the upper panel (lower panel). ChIP analysis was performed with antibodies recognizing Smad3, Smad3/4, Smad4-DNA complex (g) MDA-MB-231 cells expressing endogenous mutant p53 and (h) H1299 cells stably expressing p53-R175H were incubated in the presence or absence of 5 ng ml⁻¹ TGF-β for 24 h and ChIP analyses were performed. (i) H1299 cells were transfected with Smad3/4, p53 or p53-R175H expression plasmids alone or in combination of Smad3/4 and p53 (Smad3/4 and p53-R175H), and then stimulated with 5 ng ml⁻¹ TGF-β for 24 h. Nuclear extracts were prepared and subjected to EMSA analysis with ³²P-end-labelled probes corresponding to cognate SBE box from the REGγ promoter. *Blocking of Smad3/4 complex formation by mutant p53-R175H. (j, k) Mutant p53 reverses TGF-β induced repression of the REGγ gene through p300. (j) H1299 cells expressing either E.V or p53-R175H, and (k) MDA-MB-231 cells were stimulated with 5 ng ml⁻¹ TGF-β for indicated time points, and subjected to ChIP analysis with indicated antibodies.

Supplementary Fig S7e). Strikingly, REGγ decreased p53 protein expression in the presence or absence of ETO, whereas REGγ knockdown further enhanced p53 protein levels in ETO-treated cell lines (Fig. 7fg), reflecting a regulation of cell cycle progression in a p53-dependent manner. In conclusion, these data show that REGγ depletion decreases drug resistance and sensitizes the cancer cells to chemotherapeutic agents. In addition, overexpression of REGγ affects the tumour suppressive activities of p53 and TGF-β signalling in cancer cells to enhance cancer progression.

Knockdown of REGγ arrests growth and cell cycle progression. Next, we sought to examine the role of REGγ on cellular growth...
and cell cycle transition. To this end, we performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in HCT116p53 +/+ and HCT116p53 −/− cell lines, in which REGγ was stably knocked down. Intriguingly, we found that in comparison with REGγ-positive HCT116p53 +/+ cell line, REGγ-expressing HCT116p53 −/− cells (SHN) had markedly enhanced cell proliferation. In contrast, REGγ knockdown (SHR) cells showed reduced proliferation in both HCT116p53 +/+ and HCT116p53 −/− cell lines (Fig. 8a), suggesting that REGγ can further enhance cell proliferation in the absence of p53. Intriguingly, REGγ knockdown led to inhibition of cell proliferation in ARO (Fig. 8b) and A549 (Fig. 8c) cell lines, suggesting that REGγ promotes cell viability. Next, we assessed the proliferative role of REGγ and mutant p53 in breast cancer cell lines. Importantly, knockdown of REGγ and mutant p53 independently decreased cell growth (Fig. 8d and Supplementary Figs S7d and S9). Strikingly, cosilencing of mutant p53 and REGγ further inhibited cell proliferation in breast cancer cell lines (Fig. 8d). These data indicate that REGγ has an important role in cell proliferation of these cancer cell lines in response to p53 signalling.

Next, we aimed to examine the effect of REGγ on cell cycle progression in cancer cell lines. Fluorescence-activated cell sorting analyses showed that overexpression of REGγ enhanced
the S (DNA synthesis) and G2/M (mitotic) phase (Fig. 8e). Conversely, depletion of REGγ attenuated transition of G0/G1 cells to S and G2/M phases, which are important for cell growth (Fig. 8f–h). These data indicate that REGγ promotes cell growth via enhancing DNA synthesis in cancer cells.

REGγ expression correlates with mutant p53 in cancer tissues. Next, we examined REGγ expression in several human cancer cell lines. In contrast to p53-expressing cancer cell lines, we observed higher expression of REGγ in mutant p53-expressing cells (Fig. 9a, left panel). Importantly, western blot analysis also showed higher level of REGγ in Smad4-null cells. However, cancer cell lines expressing Smad4 had reduced level of REGγ (Fig. 9a, right panel). We observed similar results by analysing REGγ transcript in these cancer cell lines (Fig. 9b,c).

To further understand the biological relevance of our findings discussed above in tumour development, we evaluated the correlation between REGγ and mutant p53 expression by immunohistochemistry (IHC) analysis of 101 human cancer tissues. We detected a higher percentage of REGγ-positive rates in specimens with p53 overexpression compared with p53-negative groups, suggesting a positive correlation of mutant p53 with REGγ (Fig. 9d). The statistical analysis between REGγ and mutant p53 is shown in Fig. 9e and Supplementary Fig. S10a. We analysed the correlation between REGγ and mutant p53 from the same sets of tumours by scatter and agreement plots. These plots indicated that these two proteins are highly associated with each other in the same sets as that of the tumours (Fig. 9f and Supplementary Fig. S10b).

Furthermore, we performed bioinformatic analysis to check the effect of REGγ overexpression on p53/TGF-β signalling...
Depleted cells but not in control cell lines. Data are representative of three biological repeats with mean analysed by fluorescence-activated cell sorting (FACS) analysis. Data are representative of three biological replicates with mean.

In this report, we investigated the previously unknown mechan-ism for regulation of the REG-20S proteasome pathway in cancer cells during tumour development. This study indicates differential regulation of REGγ–20S proteasome pathway by p53/TGF-β signalling and mutant p53 proteins via REGγ in tumour cells. Our results provide the first example that anomaly in p53/TGF-β tumour suppressive signalling deregulates REGγ-mediated ubiquitin/ATP-independent proteasome pathway during tumorigenesis (Fig. 10a). REGγ has been found to promote degradation of p53 by acting as a coactivator to promote MDM2-mediated p53 ubiquitination. Depletion of REGγ has been shown to lead to increased p53 protein levels in several cancer cell lines. Together with previous findings, the present study provides a previously unknown model, in which an auto-regulatory feedback loop exists between p53 and REGγ. We believe that when p53 is elevated to elicit its biological functions, such as apoptosis, the activity of the REGγ–proteasome pathway will be inhibited to avoid p53 degradation and cell proliferative effects.

It is generally believed that the Smad-dependent pathway is involved in TGF-β tumour suppressive functions, whereas activation of Smad-independent pathways is coupled with loss of tumour suppressor function of TGF-β, which is important for its pro-oncogenic effects. Elevated expression of REGγ in both colonic adenoma and invasive cancers suggest that REGγ may have important roles during carcinogenesis. Thus, inhibition of the REGγ–20S proteasome pathway by TGF-β may reduce the risk of cancer development. Combinatorial control of gene expression by p53 and Smad provides a new tier in the regulation of TGF-β gene responses. Our data indicate that neither p53 nor Smads serves as a DNA-binding platform for each other, yet their coexistence greatly augment their binding. Thus, cooperation of p53/TGF-β may fine-tune cytostatic programme in cells by cooperatively regulating the REGγ–20S proteasome pathway.

**Discussion**

In this report, we investigated the previously unknown mechan-ism for regulation of the REGγ–20S proteasome pathway in cancer cells during tumour development. This study indicates differential regulation of REGγ–20S proteasome pathway by p53/TGF-β signalling and mutant p53 proteins via REGγ in tumour cells. Our results provide the first example that anomaly in p53/TGF-β tumour suppressive signalling deregulates REGγ-mediated ubiquitin/ATP-independent proteasome pathway during tumorigenesis (Fig. 10a). REGγ has been found to promote degradation of p53 by acting as a coactivator to promote MDM2-mediated p53 ubiquitination. Depletion of REGγ has been shown to lead to increased p53 protein levels in several cancer cell lines. Together with previous findings, the present study provides a previously unknown model, in which an auto-regulatory feedback loop exists between p53 and REGγ. We believe that when p53 is elevated to elicit its biological functions, such as apoptosis, the activity of the REGγ–proteasome pathway will be inhibited to avoid p53 degradation and cell proliferative effects.

It is generally believed that the Smad-dependent pathway is involved in TGF-β tumour suppressive functions, whereas activation of Smad-independent pathways is coupled with loss of tumour suppressor function of TGF-β, which is important for its pro-oncogenic effects. Elevated expression of REGγ in both colonic adenoma and invasive cancers suggest that REGγ may have important roles during carcinogenesis. Thus, inhibition of the REGγ–20S proteasome pathway by TGF-β may reduce the risk of cancer development. Combinatorial control of gene expression by p53 and Smad provides a new tier in the regulation of TGF-β gene responses. Our data indicate that neither p53 nor Smads serves as a DNA-binding platform for each other, yet their coexistence greatly augment their binding. Thus, cooperation of p53/TGF-β may fine-tune cytostatic programme in cells by cooperatively regulating the REGγ–20S proteasome pathway.
Figure 9 | REGγ shows a positive correlation with mutant p53 in multiple cancer tissues and cell lines. (a) Western blot analyses of cellular REGγ protein levels in p53- and mutant p53-expressing cancer cells, which is correlated with the p53 status (left panel). Smad4-null cancer cells show more REGγ protein levels in comparison with Smad4-containing cells (right panel). (b) Quantitative RT-PCR analyses of cellular REGγ mRNA levels in four different groups of cancer cells, which are correlated with the p53 status. Error bars show the mean ± s.d. from three technical replicates (two-tailed Student’s t-test, *P<0.05, **P<0.005). (c) Total RNA was extracted from each of the four different cancer cell lines containing Smad4-null and Smad4-expressing cells. RT-PCR analyses were performed to measure the REGγ mRNA levels. Error bars show the mean ± s.d. from three technical replicates (two-tailed Student’s t-test, *P<0.05, **P<0.005). (d) Mutant p53 positively correlates with the REGγ. IHC analysis of the multiple cancer tissues, which express different groups of mutant p53 proteins, displayed positive correlation with the REGγ overexpression. Scale bars, 50 μm (shown on × 40 images). (e) Statistical analysis of REGγ and mutant p53-positive cancer tissues. (f) Scatter plot for REGγ and mut-p53 correlation in the same sets of tumours. The scores were put into the plot using the Bland–Altman plot standard method. (g) Bioinformatics analysis of lung and colon cancers, in which overexpression of REGγ negatively correlated with the p53 pathway and TGF-β receptors expression. Pearson’s correlation coefficient was used as a measure of correlation between REGγ and its potentially related genes. Pearson’s correlation analysis was conducted using ‘R programme’ on data set with significant overexpression of REGγ.

GOF effects of mutant p53 is associated with poor prognosis in cancers. In this study, we show that mutation of p53 enhances REGγ transcription in breast and colon cancer cells. In addition, mutant p53 antagonizes Smad-dependent inhibition of REGγ expression in response to TGF-β by preventing the recruitment of the Smad/N-Cor complex. The accumulated mutant p53 can function as an activator of the REGγ–20S proteasome pathway. Strikingly, mutant p53 recruits p300 on one hand and, on another hand, blocks Smad protein binding. These findings suggest a novel bipartite mechanism by which mutant p53 reprograms transcriptional activation of proteasome activator in cancer cells (Fig. 10b).

Our study highlights the link between p53/TGF-β signalling and the 20S proteasome pathway via REGγ. Our results indicate that both p53 and TGF-β/Smad signalling can inhibit the REGγ–20S proteasome pathway to prevent degradation of important tumour suppressor proteins such as p53/p21/p16. Our data also suggest that REGγ-expressing cells are more resistant to anti-cancer drugs, and knockdown of REGγ decreases chemoresistance in cancer cells. Importantly, we also show that REGγ can attenuate the antiproliferative function of TGF-β/Smad signalling. The opposite behaviour of REGγ in cell proliferation and cell cycle regulation upon silencing p53 or mutant p53 reflect the differential response to distinct p53 signalling and further validate the regulatory mechanisms. Intriguingly, our data also suggest that overexpression of REGγ accelerates DNA synthesis via enhancing S and G2/M phase during tumour development.

Strikingly, our IHC analyses show a positive correlation between mutant p53 and REGγ in the rectum, gastric, renal, ovary and colon cancer tissues. Similarly, Smad4-null breast
cancer cell lines reveal higher expression of REGγ. Importantly, these analyses indicate that targeting the REGγ–20S proteasome pathway for cancer therapy may be helpful for human tumours having abnormal Smad3/p53 proteins status. In addition to this, our bioinformatics data also show that overexpression of REGγ in lung and colon cancer tissues negatively regulates tumour suppressor p53 and TGF-β signalling. Before our studies, no satisfactory mechanism has been proposed for the REGγ–20S proteasome pathway regulation during cancer progression, even though growing evidence suggests that the REGγ–20S proteasome pathway is involved in cancer progression.

**Methods**

**Plasmids and transfection.** pcDNA3.1-p53, pcDNA3.1-R175H, pcDNA3.1-R282W, pcDNA3.1-R248W, pcDNA3.1-TA-p63 and pcDNA3.1-TA-p73 were constructed. prK8-Smad2, prK5-Smad3 and prK5-Smad4 were kindly provided by Dr Xin Hua Feng, Baylor College of Medicine. H1299, HeLa, 293T, HaCaT, HCT116 p53+/− and UMSCC-1 cell lines were transfected with LipoFectamine 2000 (Invitrogen) following manufacturer’s protocol.

**Antibodies.** The following antibodies were used in western blotting. EMSA assay, ChIP analysis and IHC experiments: Anti-p53 (DO-1, Santa Cruz), anti-p53 (FL393, Cell Signalling Technology), anti-Smad3 (Cell Signalling Technology), anti-Smad4 (Santa Cruz Biotechnology), anti-Smad2 (Santa Cruz Biotechnology), anti-REGγ (Invitrogen), anti-p-Smad3 (Cell Signalling Technology), anti-p21(BD Pharmingen), anti-β-actin (Santa Cruz), anti-N-CoR (Abcam), anti-HDAC1 (Abcam), anti-mSin3a (Abcam), anti-Me-H3K9 (Abcam) and anti-p300 (Santa Cruz).

**Cell culture and treatments.** HaCaT, 293T, MCF-7, HepG2, ARO, H1299, A549 were purchased from ATCC. UMSSC-1 (empty vector, p53 and R175H)-expressing tumour suppressor proteins and, subsequently, inhibit cancer progression, cell proliferation and decrease drug resistance in cancer cells. (a) Novel GOF of mutant p53: mutant p53 exerts bipartite mechanism to enhance the REGγ–20S proteasome pathway in cancer cells. On one side, mutant p53 binds and recruits p300, a coactivator, and on another side block the recruitment of the Smad3/N-CoR complex formation on REGγ promoter in response to TGF-β. The net gain of this mechanism is to enhance the REGγ–20S proteasome pathway via REGγ in cancer cells to accelerate cancer progression, promote cell proliferation and increase drug resistance during tumour development.

**ChIP assay.** After treatment with TGF-β/ν/Nutlin-3, nuclear proteins were cross-linked to genomic DNA by adding formaldehyde for 10 min directly to the medium to a final concentration of 1%. Crosslinking was stopped by adding glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature on a rocking platform. The medium was removed and the cells were washed twice with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4 and 8.1 mM Na2HPO4, pH 7.2). The cells were collected by scraping in ice-cold PBS supplemented with a protease inhibitor cocktail. After centrifugation, the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors and 50 mM Tris–HCl (pH 8.1)) and the lysates were sonicated to result in DNA fragments of ~200–1,000 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors and 16.7 mM Tris–HCl (pH 8.1)). Nonspecific background was removed by incubating the chromatin resuspension with a salmon sperm DNA/protein A agarose slurry for 30 min at 4°C with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated with 3 μg of indicated antibodies overnight at 4°C with rotation. The immunocomplexes were collected with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol.
using glycogen as a carrier. PCR amplification of the genomic fragments was performed with specific primers flanking putative binding sites on the REG promoter. The PCR products were separated by electrophoresis through 2% agarose. The primer sequences are described in Supplementary Table S2.

Immunohistochemistry. Paraffin-embedded sections (3 μm thick) of different tumours and adjacent normal tissues were used to perform IHC reaction. Tissue sections were deparaffinized with xylene and dehydrated with sequential washes of 100, 95 and 70% ethanol. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol for 30 min and then washed in PBS. Antigen retrieval was achieved using a pressure cooker heating in retrieval solution, pH 6, at 125°C for 4 min, followed by a 20-min cool-down period at room temperature. Slides were then incubated with anti-p53 antibody (1:300) dilutions and anti-REG antibody (1:500) dilutions at 4°C overnight. Next, the slides were rinsed three times in PBS and incubated in biotinylated rabbit anti-rabbit secondary antibody (1:50) for 1 h and then incubated with anti-p53 antibody (1:300) dilutions and anti-REG antibody (1:50) for a further 1 h. Immunohistochemistry-positive microvessels and corresponding adjacent normal tissues were visualized as brownish red in Reg immunohistochemistry sections. All procedures were performed in quadruplicate and were repeated three times. Primer sequences are described in Supplementary Table S4.

Preparation of total cell extract and western blot analysis. Cells were washed with PBS and treated with an extraction buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl and 1 mM EDTA) supplemented with 1 mM PMSF, 1 mM sodium orthovanadate (Na3VO4), 0.1 mM DTT, 0.4 μg/ml leupeptin/pepsatin). Cell extract was stored at −20°C until required. Protein samples were subject to electrophoresis in 10% SDS–PAGE. Separated proteins were electroblotted to nitrocellulose membranes (Bio-Rad) and the blot was blocked for 1 h at room temperature with blocking buffer (0.1% PBS with Tween-20 with 5% fat-free, dried milk powder). The blot was then incubated with primary antibodies, (1:10,000 dilutions) at 4°C overnight. The blot was washed three times with 0.1% Tris-buffered saline with Tween-20 and incubated with secondary antibodies (mouse, rabbit, 1:15,000 dilutions) for 1 h. The blot was washed again three times and exposed to Odyssey LI-COR-scanner. The full-length membranes are shown in Supplementary Figs S11–S18.

MTT assay. Cell viability was assessed with an MTT assay in replicates. Cells were seeded in 96-well plates at 2.5 × 10^3 cells per well and incubated in 10% fetal bovine serum supplemented with DMEM for 24 h. After that, cells were treated with ETO/TGF-β for indicated time points. Controls received dimethylsulphoxide vehicle at a concentration equal to that in drug-treated cells. Next, drug-containing medium was replaced with 200 μl of 10% fetal bovine serum supplemented with DMEM containing 0.5 mg/ml MTT, and cells were incubated in the CO2 incubator at 37°C for 2 h and absorbance (490 nm) was measured and analysed.

Cell cycle analysis. Cell cycle analysis was carried out using DNA contents with flow cytometry. Cells were fixed in ice-cold 70% ethanol, incubated overnight at −20°C and stained with propidium iodide/Triton X-100 containing RNAse A solution for 15 min at 37°C. Cell cycle analysis was performed using BD Cell Cycle Express 2.2.1.8.4.

Trp53-like assay. Cells were plated in 96-well plates. After 24 h, cells were treated with TGF-β/ETO for an additional 24 h. Next, 100 μl per well Proteasome-Glo trp53-like cell-based reagent (catalogue number G8760, Promega) was added and mixed by plate shaking. Luminescence was measured using a luminometer, 15 min after adding the reagent. The Proteasome-Glo cell-based reagents contains a specific luminogenic proteasome substrate in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. It contains peptide-substrate Z-LRR-aminoluciferin (Z-Leucine-Arginine-Arginine-aminoluciferin) for determination of trp53-like activity of 20S proteasome.

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