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STAT5A is regulated by DNA damage via the tumor suppressor p53

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ABSTRACT

Here we report that the STAT5A transcription factor is a direct p53 transcriptional target gene. STAT5A is well expressed in p53 wild type cells but not in p53-null cells. Inhibition of p53 reduces STAT5A expression. DNA damaging agents such as doxorubicin also induced STAT5A expression in a p53 dependent manner. Two p53 binding sites were mapped in the STAT5A gene and named PBS1 and PBS2; these sites were sufficient to confer p53 responsiveness in a luciferase reporter gene. Chromatin immunoprecipitation experiments revealed that PBS2 has constitutive p53 bound to it, while p53 binding to PBS1 required DNA damage. In normal human breast lobules, weak p53 staining correlated with regions of intense STAT5A staining. Interestingly, in a cohort of triple negative breast tumor tissues there was little correlation between regions of p53 and STAT5A staining, likely reflecting a high frequency of p53 mutations that stabilize the protein in these tumors. We thus reveal an unexpected connection between cytokine signaling and p53.

1. Introduction

Mutations in the p53 tumor suppressor are found in around 50% of human cancers [1]. In experimental tumor models, p53 prevents tumorigenesis by initiating a plethora of anticancer mechanisms, which include cell senescence and apoptosis [2]. Tumors that evolved with a normal p53 gene are thought to have mutations in genes that activate p53 or in p53 targets genes that play a role in tumor suppression [3–5]. It is likely that the tumor suppressor activities of p53 depend on the contextual action of many of its target genes since it has been difficult to experimentally reproduce the phenotype of p53 mutations by disabling some of its targets in isolation. For example, p53-dependent apoptosis requires PUMA in most tissues but PUMA-null mice are not as tumor prone as p53-null mice [6]. In fact, in some contexts, PUMA and apoptosis appear to have oncogenic functions [7]. Also, mice expressing a p53 variant defective in regulating apoptosis revealed that chromosome stability is critical for the tumor suppression functions of p53 [8]. The ability of p53 to regulate senescence is also important for tumor suppression [9–11], and most importantly oncogene activation specifically enhances the ability of p53 to induce this response [10].

The mechanisms explaining the activation of p53 by oncogenes include the DNA damage response [12–14]. Oncogenes activate p53 by causing DNA damage [15,16] but also by inducing the expression of proteins that sense the DNA damage, amplify the signal and activate antitumor responses [17,18]. Lack of these proteins can impair the response to oncogene-induced DNA damage and disable the induction of p53 and tumor suppression [16,19,20]. To sustain such a complex tumor suppressor response based on DNA damage signaling it is likely that p53 itself contribute to reinforce the pathway by further inducing the expression of genes that play a role in the DNA damage response. For example, p53 induces the tumor suppressor PML [21], which helps to sustain the DNA damage response by preventing the expression of E2F targets involved in DNA repair [17]. Like PML, the transcription factor STAT5A is sufficient to induce senescence and a DNA damage response [12,18,22] but the regulation of its expression is still poorly understood.

Here we report that STAT5A is a p53 target gene capable of regulating the expression of antiapoptotic genes in cells where p53 is activated. STAT5A was originally identified as the factor conferring response to prolactin in the mammary gland [23] and is essential

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for development and cell differentiation in this tissue in mice [24,25]. Since STAT5A inhibits apoptosis and promotes mammary gland differentiation and cellular senescence we propose that it is a critical factor in deciding which tumor suppressor response is activated by p53. In addition, we reveal that in epithelial cells from normal mammary tissue STAT5A and p53 expression correlate closely while this relationship is absent in a cohort of triple negative breast tumors, where p53 is often mutated [26].

2. Materials and methods

2.1. Cell lines, culturing, plasmids and retroviral transduction

LNCaP (ATCC), MCF7, H1299, and packaging cells Amphotropic-Phoenix were cultured in high-glucose DMEM media (Invitrogen) supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin G-streptomycin sulfate (Life Technologies). All cell lines were grown in the presence of 5% CO_2 at 37 °C.

The wt-p53 and dominant negative mutant p53 (dn-p53) were described in [27] and shRNA against p53 (sh-p53) in [28]. Retroviral transduction was carried out as described [29]. Infected cell lines were selected with 2–5 μg/ml of puromycin (Sigma) and 100–200 μg/ml of hygromycin (Sigma) wherever applicable.

2.2. Detection of protein through western blotting and immunofluorescence

Whole cell lysates equivalent to 25–50 μg of total protein were resolved through 10% SDS–PAGE. Resolved proteins were transferred onto PVDF (Millipore) membranes and probed with specific antibodies. The proteins were visualized using HRP-conjugated secondary antibodies (Sigma) and Lumilight Plus (Amersham) chemiluminescence detection kit. The following antibodies were used: from Cell Signaling Technologies, STAT5 (#9363), Bcl2 (#2872), BclXL (#2762), Mcl-1 (#4572), p53 (IC12, #2524), p21 (2946), from Santa Cruz, STAT5A (sc-1081) and from Sigma, α-tubulin (B-5-1-2). For immunofluorescence, cell lines expressing various constructs, or treated with doxorubicin, were cultured overnight on coverslips, fixed in 3% paraformaldehyde, and immunostained with anti-p53, or anti-STAT5A antibodies, detected with the appropriate secondary antibodies, as described previously [30]. Images were acquired using a TCS SP2 confocal microscope (Leica Microsystems, Richmond Hill, Ontario, Canada) in the Queen's Cancer Research Institute and Protein Discovery and Function Facility.

2.3. Detection of mRNA through semi-quantitative RT-PCR

Total RNA was prepared using Ultraspec®RNA reagent (Biotecx Laboratories). The first-strand cDNAs was synthesized with a RevertAid®H Minus first-strand synthesis kit (Fermentas) using total RNA as the template and random primers. The primers used for the PCR amplification of *STAT5A*, *STAT5B* and *β-actin* transcripts are in SI Table 1. We measured the levels of the *β-actin* mRNA as control to verify the equal template usage for amplification and equal loading.

2.4. Bioinformatics to identify STAT5A gene promoter, potential p53 binding sites

The human STAT5A gene (Gene ID 12073) promoter (Promoter ID 17260) sequence was retrieved using Cold Spring Harbor Laboratory's Transcriptional Regulatory Elements Database (TRED) at http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchPromForm. The presence of potential p53 binding sites (PBS) in the vicinity of STAT5 promoter was identified using three different software programs; p53MH [31]; p53Scan [32] and a Kd based high affinity p53 binding predictor method [33].

2.5. Construction of luciferase reporter vectors containing p53 binding site and luciferase reporter assay

PBS1 and PBS2 each were amplified on about 2 Kb fragment (flanked by 1 Kb context sequence on either sides) using human genomic DNA and primers as listed in Supplementary Table 1. Subsequently, these were directionally cloned upstream of a firefly luciferase reporter in pGL3-Promoter vector (Promega) through MluI-XhoI and KpnI-HindIII sites respectively. Cells were transfected either in 12- or 24-well format using the Metafectene (Biontex). Also included 100–200 ng of the firefly luciferase reporter plasmids and 10–20 ng of the *Renilla* luciferase reporter plasmid under the control of the housekeeping *β-globin* gene promoter. Additional treatments of the cells are indicated wherever applicable. Cells were harvested 48 h post-transfection for analysis using a Dual-Luciferase assay kit (Promega). Samples were read using an Lmax microplate luminometer (Sunnyvale, CA), and data acquisition was done by using SoftmaxPro software.

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out using LNCaP prostate cancer cells and using ExactaChIP™ kit specifically designed for p53 (R&D Systems). All the primers used for amplification of ChIP fragments are listed (in Supplementary Table 1). The *p21* specific and *actin* specific primers were used respectively as positive and negative p53-binding controls.

2.7. Immunofluorescence staining and analysis of p53 and STAT5A in breast cancer cases

A cohort of one triple positive and five triple negative breast cancers was obtained from the archived tumor tissue bank of the Department of Pathology and Molecular Medicine and Kingston General Hospital. After obtaining approval from the Research Ethics Review Board at Queen's University, a tissue microarray was constructed from formalin fixed paraffin embedded tissues, consisting of three 0.5 mm diameter cores per tissue. A control consisted of two normal mammoplasties. The TMA was double immunostained with mouse anti-p53 and rabbit anti-STAT5A antibodies detected with Alexa488 anti-rabbit (green) and Alexa567 anti-mouse (red) secondary antibodies (see antibody origin detailed above). Nuclei were stained with DAPI (blue). All staining was performed using an automated staining processor (Ventana Medical Systems, Tucson, AZ, USA) with citrate buffer pH 8, as per manufacturer's instructions. IF images (200× magnification) of whole cores were acquired from stained TMA sections using a ScanScope FL instrument (Aperio Technologies, Vista, CA) [34]. The expression of p53 within each tumor (Fig. 6 Column 2, Cases 1 and 5) was variable as indicated in the whole core merges, but generally higher than in the normal duct tissue (Column 2, case 6). We therefore performed a co-localization study which determines the overlap of p53 (red) and Stat5A (green) pixels double immunostained for each protein in the same core. Zoom-up images of selected fields of +ve p53 staining were acquired (630× magnification) for each tissue core using a TCS SP2 confocal microscope (Leica Microsystems) (Fig. 6 Columns 3–5). p53 and STAT5A immunoreactivity in each high resolution image was measured using Image Pro Analysis Software, and the degree of red/green pixel co-localization for each tumor core expressed as a Pearson correlation coefficient (PCC). Three images from 2 to 3 cores per tissue sample were analyzed. A 2-sided Student *t* test was used

to determine significance between average correlation coefficients of tumor versus normal breast tissues.

3. Results

3.1. STAT5 is induced during Ras-induced senescence in human mammary epithelial cells (HMECs)

The ras oncogene has been implicated in multiple human cancers and its transforming activity is due to the constitutive activation of multiple signaling pathways including the ERK/MAP kinase pathway and the PI3K pathway. However, in primary cells oncogenic ras induces a tumor suppressor response characterized by a permanent cell cycle arrest and the secretion of inflammatory cytokines. This response is known as oncogene-induced senescence (OIS) [35]. We characterized the gene expression profile of OIS in primary human fibroblasts and found that STAT5 was highly expressed after expression of oncogenic ras in these cells [29]. We also reported that expression of a constitutively active STAT5A allele in HMECs induced senescence [35]. To investigate whether oncogenic ras can induce STAT5A in HMECs we infected these cells with a ras-expressing retroviral vector and obtained a population of senescent cells for RNA expression analysis. As expected, the expression of the proliferation marker Ki67 was largely reduced by oncogenic ras, while the senescence-associated cytokine IL-6 and the p53 targets GADD45, p21 and PML were highly induced (Fig. 1). In addition STAT5A was also induced by oncogenic ras in this setting (Fig. 1). Since p53 modulates the expression of many senescent genes we next investigated whether p53 could influence STAT5A gene expression.

3.1.1. p53 modulates STAT5A expression and activity

We first used MCF7 breast cancer cells stably modified to under- or over-express p53. As shown in Fig. 2A, STAT5 expression was decreased by shRNA against p53 or a dominant negative p53 allele and induced by enforcing the expression of wild type p53 by retroviral gene transfer. The STAT5 target gene MCL1 moderately followed the expression changes in STAT5 while the expression of the p53 target p21 was similar to STAT5. The efficiency of the validated shRNA [28] to decrease p53 was assessed by immunoblotting (Fig. 2B). In compliance with the above observations, immunofluorescence staining also showed an increased level of nuclear STAT5A in MCF7 cells transfected with vector expressing wt-p53 (Fig. 2C).

To investigate whether p53-mediated induction of STAT5A was specific for the breast or it could happen in other tissues we next study prostate epithelial cells LNCaP (wild type for p53) or PC3 (deleted for both p53 alleles). We compared the expression of STAT5A in these two cell lines and found a close correlation between the expression of STAT5A and p53 in these cells (Fig. 2D). To investigate whether STAT5A expression was dependent on p53 we stably transduced LNCaP cells with retroviruses expressing a shRNA against p53 [28] and a dominant negative mutant of p53 [27]. As shown in Fig. 2E, STAT5A expression was considerably downregulated upon blocking of p53 function by both reagents in LNCaP cells. The STAT5A target gene MCL-1 [36] was significantly downregulated upon inactivation of p53 (Fig. 2E) showing a much stronger dependency on STAT5 expression than in breast cancer cells. To further investigate the regulation of STAT5A by p53 we used semi-quantitative RT-PCR assay. The expression of STAT5A mRNA was decreased after p53 knockdown and increased by overexpression of wild type p53 (wt-p53). In contrast, the related gene STAT5B was not affected by p53 modulation (Fig. 2F). We thus conclude that p53 mediated regulation of STAT5A gene expression is not specific to breast tissue.

Many p53 functions are modulated by DNA damage and p53 target genes that regulate growth arrest and apoptosis are induced by DNA damage in a p53 dependent manner [2]. Therefore we decided to investigate whether DNA damage could induce STAT5A

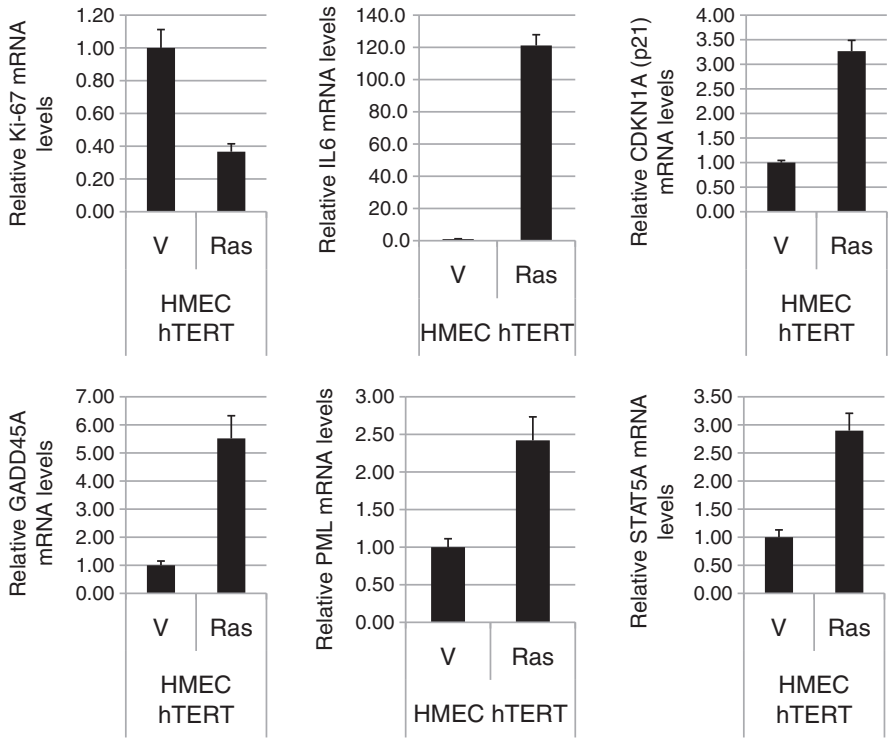


Fig. 1. Induction of STAT5A during oncogene induced senescence in HMECs. qPCR for the indicated genes in cells forced to senesce with oncogenic *ras*. Cells with an empty vector were used as control.

as well. We treated LNCaP prostate cancer cells and MCF7 breast cancer cells with doxorubicin. We found that this DNA damaging drug induced STAT5A levels and its nuclear localization in both cell lines (Fig. 3A and B). Using immunoblots we confirmed the induction of STAT5A and the expression of its targets MCL-1 and BCLXL upon DNA damage (Fig. 3C). In conjunction, these data strongly indicate that p53 controls STAT5A expression.

3.1.2. p53 binding sites regulating STAT5A gene expression

Traditionally, searching for transcription factors binding sites regulating particular genes is limited to a small region surrounding the promoter. There are no consensus p53 binding sites close to the STAT5A transcriptional start site (TSS). However, unbiased mapping of transcription factor binding sites in the chromosomes 21 and 22 revealed that many transcription factors, including p53, bind to sites far away from the TSS and often in the 3′ part of the gene [37]. For this reason we searched for p53 binding sites over a region of 10 kb upstream and downstream of STAT5A transcription start site using multiple algorithms, p53MH [31], p53Scan [32] and high affinity p53 binding predictor as described [33] (Supplementary Table 2). Both p53MH and p53scan algorithms recognized one high score p53 binding site (PBS1) 7.2 Kb upstream of the TSS. Another p53 binding site (PBS2) was predicted, by all three methods, in an Alu repeat in the 5th intron of the gene, around 9.5 Kb downstream of the TSS (Fig. 4A). PBS1 and its flanking sequences are conserved in Pan troglodytes and Macaca mulatta but no conservation of this region was found in rodents. We cloned fragments of the STAT5A locus containing these sites into the luciferase reporter vector pGL3 and evaluated its ability to confer p53-dependent luciferase expression. In the p53-null cell line H1299, transfection of wt-p53 activated transcription from reporters containing either PBS1 or PBS2 and PBS2 being the most active one (Fig. 4B and C). This stimulation was inhibited by small oligonucleotides containing the p53 binding sites PBS1 and PBS2 (Fig. 4D and Supplementary Table 1). Strikingly, in LNCaP cells only the PBS1 containing fragment conferred DNA damage responsiveness (Fig. 4E).

Next we studied whether p53 could be found bound to either PBS1 or PBS2 sequences by using chromatin immunoprecipitation assay in p53 wild type LNCaP cells with primers flanking both the PBS1 and PBS2 p53-responsive elements in the STAT5 locus (Fig. 5A). Endogenous p53 was found constitutively bound to the promoter of well-known p53 target p21 and to PBS2 but much less bound to PBS1 (Fig. 5B). Upon DNA damage stimulation, the binding to PBS1 increased while the binding to PBS2 remained unaltered (Fig. 5C). This is consistent with our finding that in these cells PBS1 but not PBS2 confer DNA damage responsiveness to a luciferase reporter (Fig. 4E).

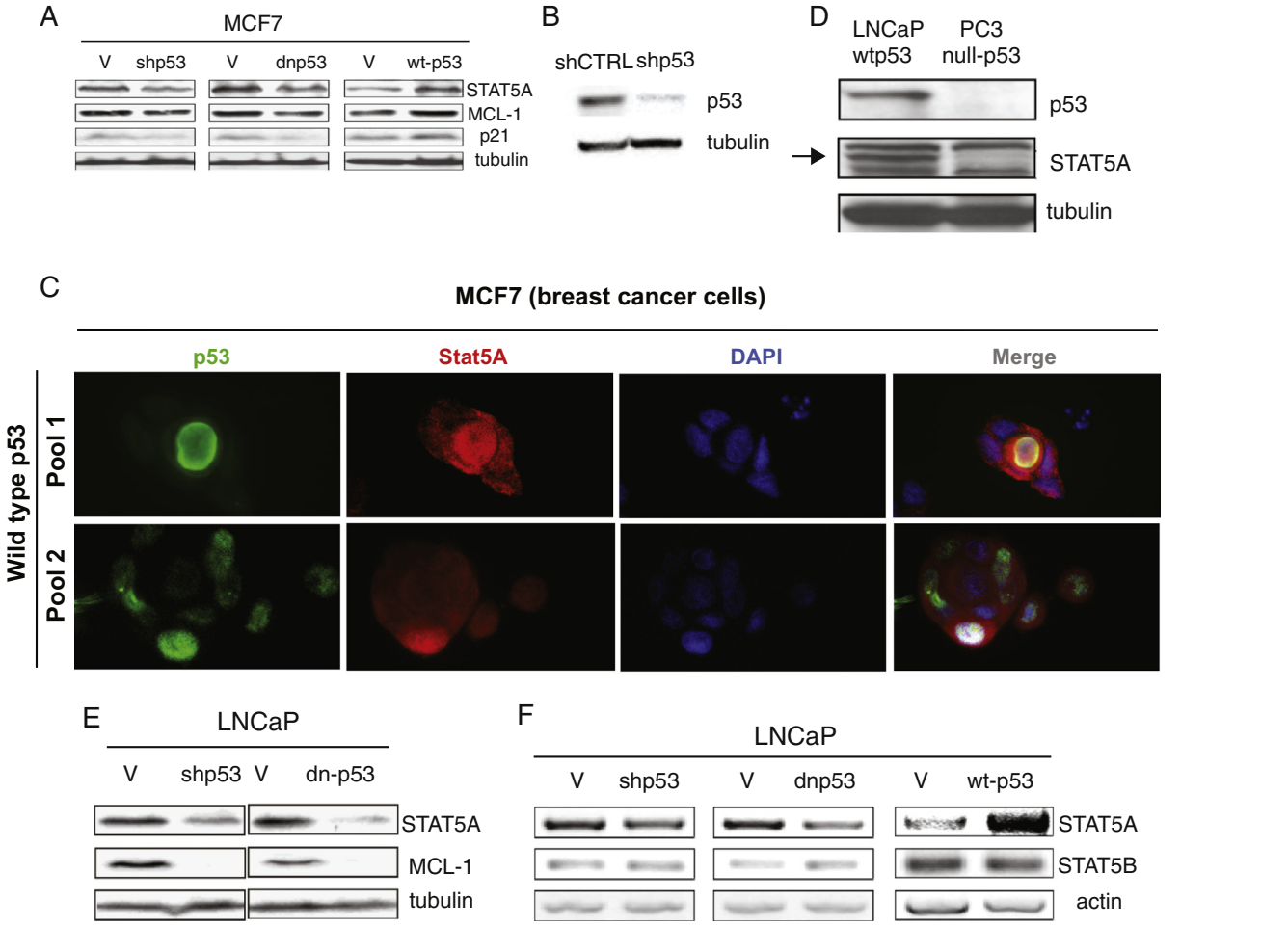


Fig. 2. Regulation of STAT5 by p53. (A) Western blot showing STAT5A, MCL-1 and p21 levels in MCF7 cells with either attenuated or exacerbated p53. (B) Western blot in MCF7 cells expressing a control shRNA or a shRNA against p53. (C) MCF7 cells were transfected with wt-p53 expression construct (plpc-p53) and subjected to immunofluorescence microscopy. DAPI was used as nuclear stain. Results from two clones are shown. (D) Western blot showing total STAT5 and STAT5A status in p53 wild type LNCaP and p53-null PC-3 prostate cancer cell lines (E) STAT5A and MCL-1 expression in LNCaP prostate cancer cell and it's derivative expressing shRNA against p53 or a dominant negative p53. (F) Semi quantitative RT-PCR for STAT5A and STAT5B in LNCaP cells expressing shRNA against p53, dominant negative (dn) p53 or wild type (wt) p53.

3.2. STAT5A expression in the breast correlates with p53 wild type levels

High expression and nuclear localization of STAT5A in breast tumors is considered a sign of good prognosis [38,39], while low STAT5A expression is associated with tumor progression and poor prognosis [40]. Since we found that STAT5A is a p53 target gene we thought that the status of STAT5A in the breast could indirectly indicate the status of p53. We thus double immunostained for both p53 and STAT5A in a TMA consisting of normal breast tissues and triple negative breast cancer cases, where p53 is often mutated [26]. Overlap of STAT5A and p53 was assessed in three images from each core, based on Pearson correlation coefficients. In normal breast lobules, the staining for p53 and STAT5A was closely matched, and most cells with p53 signals were also positive for the STAT5A signal (Fig. 6). In tumor tissues, p53 staining appeared heterogeneous and more intense than in normal breast tissue cores (Fig. 6, column 1), an indication of the presence of mutant p53 [41,42]. Furthermore, the correlation between STAT5A and p53 staining in the tumor regions was rarely seen (Fig. 6). In contrast, significant STAT5A staining in adjacent stromal tissue served as an internal positive control. Statistical analysis of images from three independent fields in each patient sample strongly suggests that STAT5A and p53 are co-expressed in normal tissues, but rarely in the cases of advanced breast cancer used in this study (Fig. 6B). This difference was highly significant ($p = 6.34E{-}06$; Student T test).

4. Discussion

Tumor suppression is a continuous physiological response to the cellular changes often triggered by cancer causing mutations. This response is very powerful in normal cells but greatly attenuated in cancer cells due to mutations and/or epigenetic changes that inactivate several tumor suppression pathways. In normal cells, oncogenes activate p53 via several pathways that together confer robustness to the tumor suppression response [15,43]. Inactivation of tumor suppressors allows tumor progression but the degree of malignancy likely depends on the extent of inactivation of all cellular anti-tumor mechanisms. The p53 tumor suppressor is the most frequently inactivated gene in human cancers [44]. p53 acts as a transcription factor and exerts tumor suppression via the action of a combination of p53 target genes [45]. The plethora of functions potentially carried out by each known p53 target gene not always explains the tumor suppressor activities of p53, indicating that the overall response is context dependent [45] as may involve unknown additional pathways. Here, we identify the transcription factor STAT5A as a novel p53 target gene. We found that STAT5A levels are induced in cells by DNA damage in a

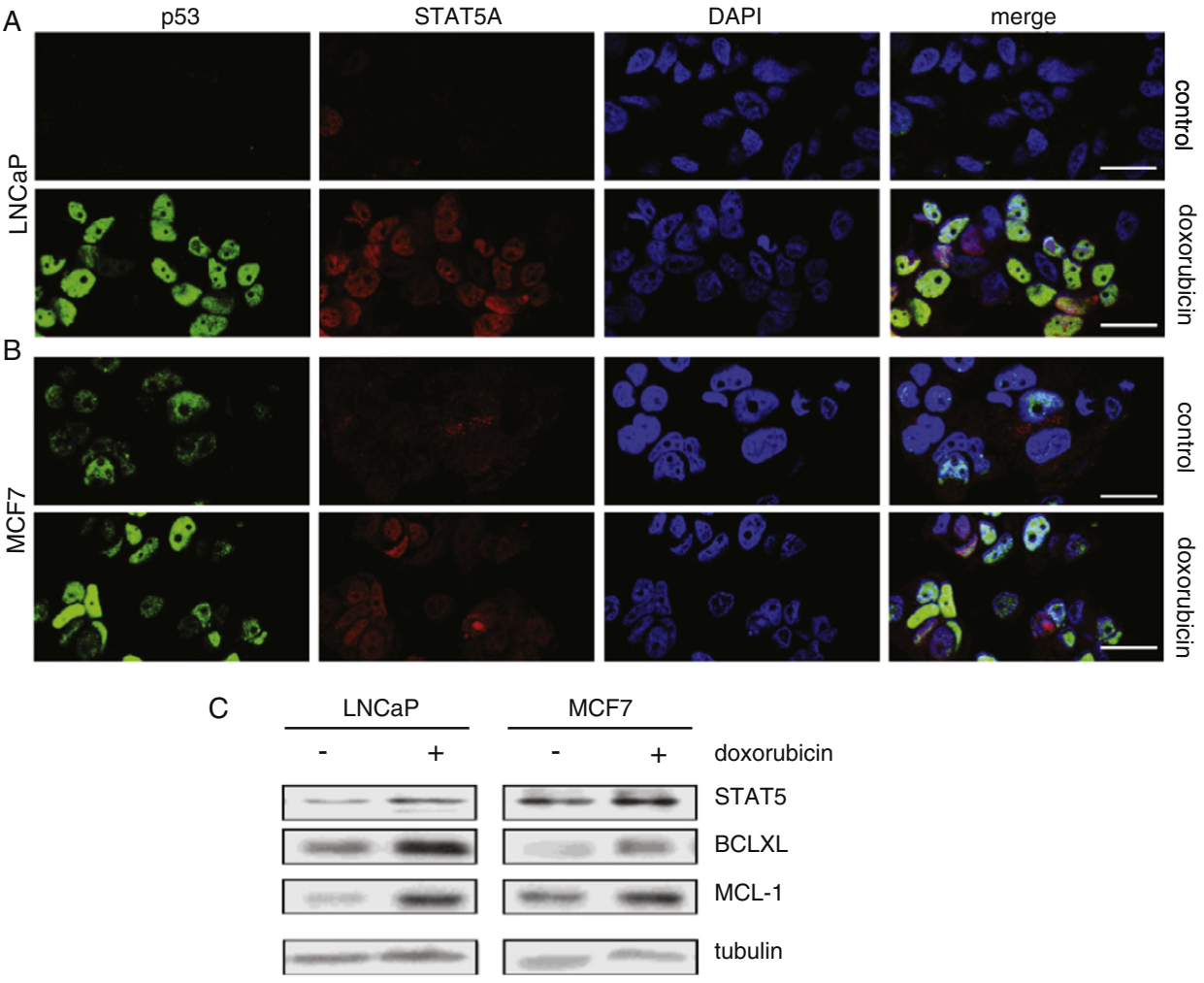


Fig. 3. The DNA damaging drug doxorubicin induces STAT5A expression. (A and B) Immunofluorescence staining for p53 and STAT5A in LNCaP prostate (A) and MCF7 breast (B) cancer cell lines treated with either 300 ng/ml doxorubicin or vehicle for 24 h. (C) Western blot analysis of STAT5 level/function in LNCaP prostate and MCF7 breast cancer cells either treated (+) or not treated (−) with doxorubicin 300 ng/ml for 24 h.

p53 dependent manner or in experimental conditions where p53 levels are increased both in prostate and breast cancer cell lines. Two p53 binding sites were identified in the STAT5A gene; one in the promoter region and one in the 5th intron. Binding of p53 to these sites was demonstrated by ChIP assay. Altogether, the data indicates that STAT5A may play a role in shaping the tumor suppression responses orchestrated by p53 and establish an unanticipated connection between cytokine signaling and the p53 response.

STAT5A is also a transcription factor, and like p53 regulates a plethora of target genes that can potentially accelerate or inhibit tumor progression [46]. In some cancer types, including prostate [47–49] and haematopoietic cancers [50–52] STAT5 plays a prosurvival/progression role. The effect of STAT5 activation in tumors is therefore context dependent and it may accelerate tumor progression in the prostate while limiting it in the breast [40,46]. The combined action of STAT5A and p53 could determine the kind of tumor suppressor response regulated by p53. Since p53 can induce

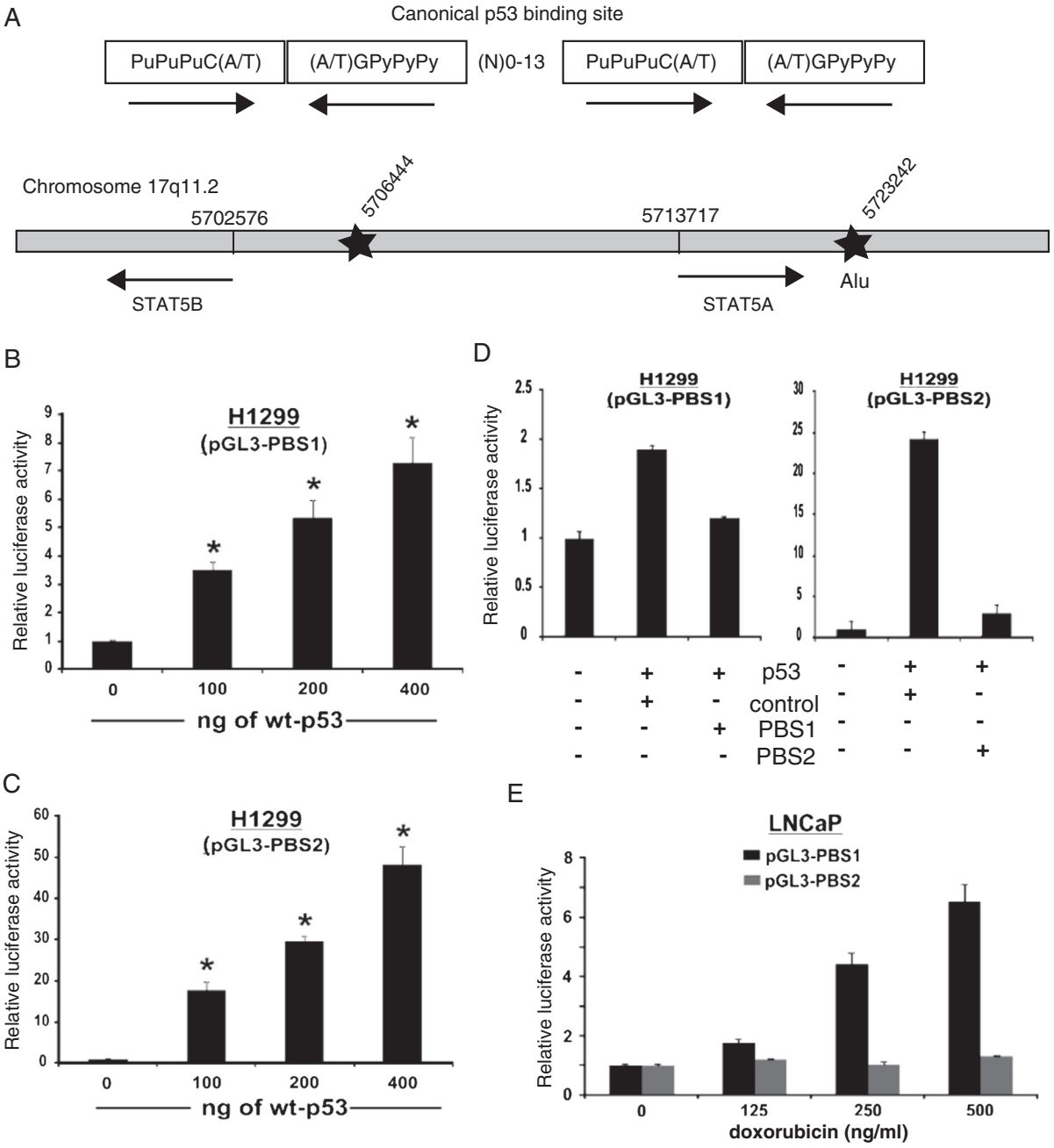


Fig. 4. Transactivation of *STAT5A* gene promoter by p53 and DNA damaging agent doxorubicin. (A) Shown on the top a canonical p53 binding site sequence. '*N*' represents a spacer sequence of any nucleotide and the allowable nucleotide length between 0 and 13 (subscript). The bottom panel shows a schematic representation of *STAT5* locus (not to scale) on Chromosome 17q11.2. Also shown are the location of PBS1 in the intergenic region of *STAT5A* and *STAT5B* and PBS2 located in an Alu repeat in the 5th intron of the *STAT5A* gene. The numerals indicate the actual location of various elements. (B and C) Relative luciferase activity of STAT5 promoter reporters in cells transfected with different amounts of p53 expressing vector. (D) Inhibition of p53 transcriptional activity by oligonucleotides containing the p53 response elements present in the *STAT5A* gene. Cells were also cotransfected with 100 ng of wt-p53 and 1000 fold molar excess of a control oligonucleotide duplex or double stranded-oligo containing PBS1/PBS2 as indicated. (E) PBS1 but not PBS2 confers responsiveness to DNA damage in LNCaP cells. Error bars represent SD of three independent measurements and $*$ denotes a p value <0.05.

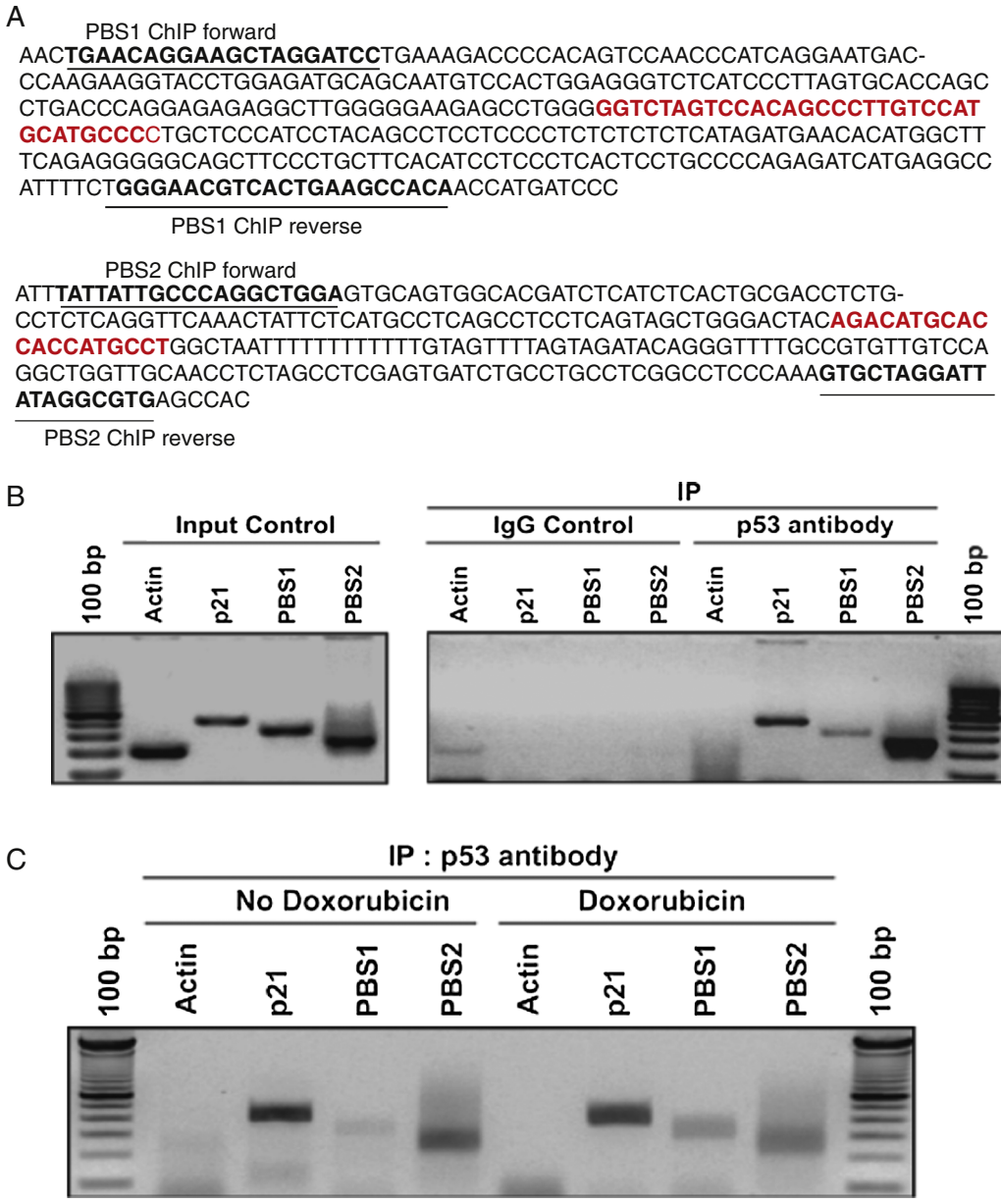
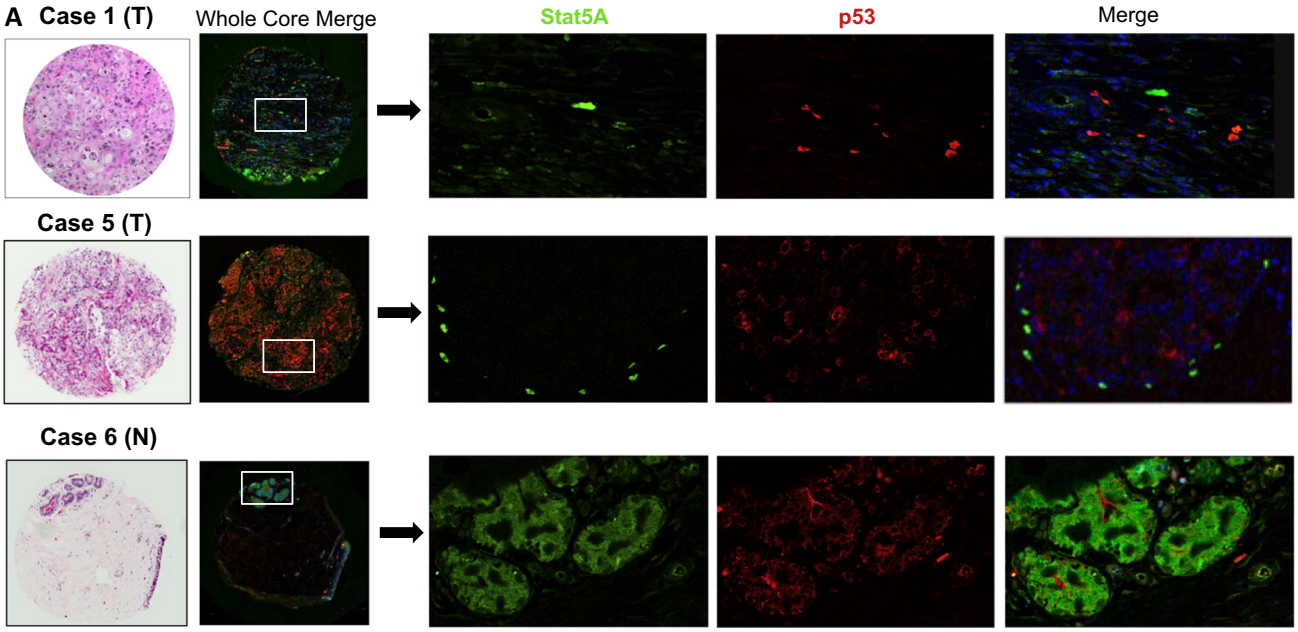


Fig. 5. *In vivo* occupancy of p53 to the p53 binding elements in the *STAT5A* gene. (A) Sequences flanking the p53 binding sequences (PBS) found in the STAT5A gene. The location of the primers used for the PCR amplification of immunoprecipitated chromatin is indicated. (B) Chromatin immunoprecipitation with anti-p53 and IgG control antibodies in LNCaP cell extracts. (C) Chromatin immunoprecipitation done using LNCaP either treated or not treated with p53 activating drug doxorubicin. *Actin* and *p21* promoter specific primers were used respectively as negative and positive p53 binding control.

apoptosis and STAT5A can block this event, it is plausible that, in tissues or cell line-based models where p53 induces STAT5 expression, cell cycle arrest or senescence will be the preferred tumor suppressor mechanism. Further supporting this idea, STAT5 activation is sufficient to induce the expression of genes that contribute to cellular senescence such as p21, SOCS1 and PML [12,18,35].

The value of p53 detection to assess the prognosis of human cancers has been limited. One factor affecting the interpretation of the clinical data is that most mutations that inactivate p53 stabilize the protein. Hence, it is very difficult to make conclusions about the p53 status from immunohistochemistry data [42]. Ideally, p53 target genes can help in the analysis because the p53 pathway, if intact, must translate into high expression of p53 target genes [53]. The expression and activation level of STAT5A has been considered a factor of good prognosis in breast tumors [38–40,54–57]. Hence, it is plausible that STAT5A levels and activity represent in some breast tumors a sign of ongoing tumor suppression and p53 activity. In normal breast epithelial tissue our results demonstrated that p53 and STAT5A levels closely match. However, in a cohort of triple negative breast tumor tissues, high p53 expression correlated poorly with STAT5A positivity, suggesting a dysfunctional p53 pathway in these tumors. The number of patients available in our cohort limits our results. To further strength this point we used the web server Oncomine and obtained data from four independent studies showing that STAT5A is generally reduced in breast tumors [58]. The first study includes 593 samples from the TCGA database across multiple breast cancer types and shows a clear reduction in STAT5A expression in almost every breast cancer subtype when compared with normal breast tissue (Fig. 1A in [58]). The second study (Zhao Breast) shows data from 64 samples



B

Colocalization analysis of p53 and Stat5A							
Case	Tumor (T) /normal (N)	ER/PR/HER 2	Image A	Image B	Image C	Mean Pearson Coefficient	
1	T (15345)	-/-/-	0.078	0.113	0.021	0.071±0.046	
5	T (4817)	+/+/+	0.068	0.092	0.081	0.080±0.012	
2	T (11017)	-/-/-	0.289	0.156	0.198	0.126±0.068	
3	T (20620)	-/-/-	0.147	0.168	0.188	0.168±0.026	0.152 ± 0.082***
8	T (110)	-/-/-	0.214	0.127	0.182	0.174±0.044	
9	T (17568)	-/-/-	0.328	0.279	0.277	0.295±0.029	
6	N (1713)	+/+/+	0.457	0.273	0.656	0.462±0.191	
7	N (3242)	+/+/+	0.413	0.439	0.325	0.434±0.065	0.530 ± 0.096

Fig. 6. Colocalization analysis of p53 and Stat5A in human malignant versus normal breast tissues. (A) A cohort consisting of one ER + PR + Her2 + and five triple negative breast tumors was immunostained on a TMA (three 0.5 mm dia. cores/tumor) with anti-STAT5A (green) and anti-p53 (red) antibodies, as described in *Materials and Methods*. A control consisted of two normal mammoplasty tissues. Nuclei were stained with DAPI (blue). Images (200× magnification) of whole cores stained for IF or H&E were acquired using a ScanScope FL instrument (Aperio Technologies, Vista, CA). Zoom-up images of representative p53 positive fields were taken of each core using a TCS SP2 confocal microscope (Leica Microsystems). Cases 1 and 5 represent a triple negative and a ER + PR + Her2 + breast tumor (*T*), respectively; case 6 represents a normal (*N*) mammoplasty. ER = oestrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor-2. (B) Colocalization of immunoreactivity of p53 and STAT5A in representative images from each case was measured using Image ProAnalysis Software, and mean Pearson correlation coefficient ± SD for at least three images from each case were calculated. Statistical comparison of tumor versus normal groups is shown. ***, $p < 0.0001$ by 2-sided Student's *t* test.

confirming a downregulation of STAT5A in ductal and lobular breast carcinomas when compared to normal breast tissue (Fig. 1B in [58]). The third study from Esserman breast cohort with 130 samples shows that STAT5A expression is downregulated in all histological tumor types when compared to normal tissue (Fig. 1C in [58]). Finally, data from the Perou breast cohort shows again downregulation of STAT5A in breast cancer (Fig. 1D in [58]). Interestingly, a benign tumor (fibroadenoma) had the highest STAT5A level (Fig. 1D in [58]), consistent with the idea that benign tumors are lesions where p53-dependent senescence is activated [35].

Further studies with a larger patient cohort and known p53 mutation status will be required to validate these findings and to determine whether the degree of co-staining with p53 and STAT5A antibodies correlates with good prognosis in patients. Nevertheless, our discovery that STAT5A is a p53 target and the fact that these two transcription factors coregulate several genes such as p21 and PML, defines novel feed-forward regulatory loops with implications for tumor suppression and the response to chemotherapy.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.01.013.

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