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HCT116 cells deficient in p21^{Waf1} are hypersensitive to tyrosine kinase inhibitors and adriamycin through a mechanism unrelated to p21 and dependent on p53

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Abbreviations: Chx, cycloheximide; PARP, poy(ADP-ribose)polymerase; PI, propidium iodide

Abstract

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1. Introduction

$p21^{Waf1}$ (p21 herein after) is a member of the Cip/Kip family inhibitors of cell cycle progression. The first discovered and best studied biochemical activity of p21 is to inhibit Cdks, consistent with its activity as cell cycle inhibitor [1-4]. However, more recent studies have shown that p21 has additional functions as differentiation inducer [5] and as an inhibitor of apoptosis induced by DNA-damaging agents [6-8]. Since cancer cells can escape death induced by chemotherapeutic drugs, this latter p21 activity is of pivotal importance in human cancer therapy.

p21 is a p53 target gene and is a relevant mediator of p53-cell cycle arrest [9-11]. A role of p21 in cancer has been demonstrated in vivo, as p21-null mice develop tumours spontaneously [12] and show increased tumour susceptibility using chemical carcinogenesis [13-16]. Although there are examples of p21 acting as an apoptosis inducer [17-20], the results in most models suggest it has an apoptosis protective effect. One of these cell models consists of the human colon cancer cell line HCT116 and its isogenic derivative HCT116p21$^{-/-}$, generated by disruption of both p21 alleles through homologous recombination [21]. Work with these cell lines suggested that p21 protected from apoptosis induced by adriamycin (doxorubicin) [22]. Later on, many reports have argued for a protective role of p21 against apoptosis induced by DNA-damaging agents and other stimuli as hypoxia using the HCT116 model system (a non-exhaustive list is shown in Supplemental Table 1).

In this work we asked whether p21 also played a role in the cell death induced by non-genotoxic drugs in the HCT116 model. We chose imatinib as the main drug for our studies because it is tyrosine kinase inhibitor (with higher activity against c-Abl, Bcr-Abl and c-Kit) widely used in human cancer therapy [23,24]. We found that the cytotoxic effect of imatinib, gefitinib and adriamycin was much higher on the p21-deficient HCT116 cells than on parental cells, but, surprisingly, this hypersensitivity was due to p53 overexpression and independent from p21.

2. Material and Methods

2.1 Cell lines, transfections and mice

Colon cancer cell line HCT116 and its isogenic derivatives HCT116p21$^{-/-}$ [21] and HCT116p53$^{-/-}$ cells [22] were grown in RPMI 1640 (Invitrogen) with 8% fetal calf serum and gentamycin (80 μg/ml). HKO-MTp21 cell line was generated cotransfecting HCT116p21$^{-/-}$ cells with the pM6-p21 construct (carrying a Zn^{2+}-inducible p21) [25] and the pLPCX vector in a proportion 6:1. Transfections were carried out with Lipofectamine (Invitrogen) and transfectants were selected with 0.25 μg/ml of puromycin (Sigma). HKO-MTp21 cells were incubated with 75 μM $ZnSO_4$ to induce p21. For silencing of p21, HCT116 cells and derived cell lines were transduced with empty vector (pRetroSuper), and vector carrying two different short-hairpin constructs for human p21 (shp21B and C). The targeted sequences were AACACCTCCTCATGTACAT (shp21B) and GCGACTGTGATGCGCTAAT

(shp21C). Overexpression of p21 was achieved by adenoviral infection. HCT116p21$^{-/-}$ cells were infected with adenovirus carrying p21 gene and GFP [26] and 24 h after infection whole-cell protein extracts were analysed by immunoblot. GFP-expressing cells were above 80% in all adenoviral infections. For silencing of p53, HCT116p21$^{-/-}$ cells were transduced with pSuperRetro-shp53 virus [27] and a polyclonal transduced cell line, Hp21KO-shp53, was selected with 0.5 μg/ml of puromycin. Hp21KO-pSR are HCT116p21$^{-/-}$ cells transduced with the empty vector. HCT116p21$^{-/-}$ cells were transfected with an expression vector for pcDNA3-DDp53 [28] and, to allow selection, a vector carrying a puromycin resistance markers (pLPCX) in a proportion 6:1 (DDp53:pLPCX). Transfections were carried out with Lipofectamine and transfectants were selected with 0.5 μg/ml of puromycin. A pool of five transfectants was selected and the presence of the DDp53 isoform analysed by immunoblot. p21-deficient mice (B6;129S2-*Cdkn1a*^{tm1Tyj}/J) [9]were obtained form Jackson Laboratories.

2.2 Proliferation, annexin V binding and cell cycle assays

Proliferation assays were performed to compare the response of cells to drugs. 80,000 cells (for HCT116p21$^{-/-}$) or 50,000 cells (for the other cell lines) were plated on 12-multiwell plates and 24 h after plating, increased concentrations of imatinib (provided by Novartis), gefitinib (provided by Astra-Zeneca), adriamycin (Sigma Chemical Co.), 5-fluorouracil (5-FU) (Sigma Chemical Co.) or SU6656-001 (Biaffin) were added. After 48 h cells were stained with crystal violet (1% in methanol), scanned and subjected to densitometric analysis. Clonogenic assays were performed plating 5,000 cells on 6-multiwell plates and after one week colonies where stained as above and counted. For cell cycle analysis the cells were fixed in 90% ethanol at 4ºC, washed with PBS and incubated at 37ºC for 30 min with RNase (200 μg/ml) and 10 μg/ml propidium iodide (PI) (Sigma). The stained cells were analyzed by flow citometry on a FACScan flow cytometer (BD Bioscences) using the CellQuest software. Apoptosis was assessed by annexin V binding. Cells were treated with different imatinib concentrations for 48 h and annexin V binding was determined by flow cytometry and annexin V-phycoerythrin (BD Pharmingen).

2.3 Immunofluorescence staining, immunoblotting and protein kinase assays

Cells were fixed with paraformaldehyde and the presence of phosphorylated H2AX histone (γ-H2AX, monoclonal antibody from Upstate, ref. 05-636) or p21

(policlonal antibody C-19 form Santa Cruz Biotech.) were detected by immunofluorescence, using a FITC-conjugated secondary antibody (Dako, F0205). Samples were mounted with Vectashield (Vector) containing 4'-6-diamidino-2-phenylindole (DAPI) to stain nuclei and examined under a fluorescence microscope (Zeiss-Axioskop2). Immunoblots were performed as previously described [25]. Blots were revealed with the ECL system (GE Healthcare). Antibodies used were: anti-poy(ADP-ribose)polymerase (PARP) (H-250), anti-phospho-tyrosine (PY20), anti-actin (I-19), anti-p53 (FL-393), anti-Akt1/2 (H136), anti-c-Src (SRC2), anti-Erk2 (C14), anti-phospho-Erk1/2 (E-4), anti-CDK2 (M2) from Santa Cruz Biotech; anti-p53 phosphorylated in serines 6, 9, 15, 20, 37, 46 and 392 (ref. 9919) and anti-phospho Akt (ref. 9271) from Cell Signalling; anti-phospho-c-Src (44-660G) from BioSource; anti-c-Abl (ref. 554104) from BD Pharmingen; anti-p21 (P-184) from Sigma, and anti-α-tubulin antibody provided by Nicholas Cowan (New York University, New York). To assay CDK2 activity, protein extracts (1 mg per assay) were immunoprecipitated with 1.5 μg of CDK2 antibody (same as above) and collected on Gammabind sepharose beads (Pharmacia). Kinase activity was measured on histone H1 (Roche Biochemicals) as a substrate as described [25]. An aliquot of the immunoprecipitate was analysed by immunoblot for p21 and CDK2 expression.

2.4 Reporter-luciferase assays

p53 transcriptional activity of HCT116 and HCT116p21$^{-/-}$ was measured with three promoter-luciferase constructs: PG13-Luc, p21-Luc and MDM2-Luc, as well as the promoterless pGL2-basic vector (Promega) used as negative control. One million of cells were transfected with Lipofectamine and 1 μg of the luciferase constructs plus 0.1 μg of pRL-null for transfection normalization. After 36 h cells were lysed and luciferase activity was assayed with the Dual Luciferase kit from Promega as instructed by the manufacturer. Luciferase activity was normalized to the *Renilla* internal control. Each point corresponds to the mean of two different experiments done in duplicate. Activation is relative to the values obtained with HCT116 cells transfected with the corresponding reporter.

2.5 Gene expression analysis

HCT116 and HCT116p21$^{-/-}$ cells were untreated or treated with 15 μM imatinib for 48 h. Total RNA was extracted (RNeasy kit, Qiagen), labelled and hybridized to a

membrane macroarray containing probes for 96 apoptosis-related genes (Human Apoptosis Gene Array", SuperArray Bioscience Corporation), following the manufacturer indications. The membrane was washed and the radioactivity measured in a Molecular Imager apparatus (BioRad). For each gene the signal was normalized to the control genes in the array, and the ratio between the mRNA levels in control cells and cells treated with imatinib was calculated.

3. Results

3.1 HCT116p21$^{-/-}$ cells are more sensitive to kinase inhibitors

In view of the high sensitivity of HCT116p21$^{-/-}$ to genotoxic drugs, we took advantage of this model to determine whether p21 played a role in the response to drugs that do not directly induce DNA damage such as tyrosine-kinase inhibitors in HCT116 and HCT116p21$^{-/-}$. We tested imatinib (which shows higher activity against Bcr-Abl, c-Abl and c-Kit) and gefitinib (with highest specificity against epidermal growth factor receptor). The results demonstrated that p21-deficient cells were dramatically more sensitive against imatinib and gefitinib than parental cells. We also tested p53-deficient cells [29] and found that these cells were more resistant to the kinase inhibitors that the p21-deficient cells (Fig. 1a). As a parallel approach we performed clonogenic assays, which also demonstrated the hypersensitivity of HCT116p21$^{-/-}$ cells to protein kinase inhibitors [30] (Fig. 1b). As positive controls for cell death we used adriamycin and 5-FU and we confirmed the reported hypersensitivity to both drugs of p21-deficient cells [22,31-33] (Fig. 1a, b).

A time-course proliferation assay in response to imatinib confirmed that HCT116p21$^{-/-}$ cells were more sensitive than the parental cells. The imatinib effect on p21 deficient cells was cytotoxic as growth did not resume upon removal of the drug (Fig. 1c). The results also showed that, in the absence of drugs, HCT116p21$^{-/-}$ cells grow ~five-fold slower than parental cells. The cytotoxic effect of imatinib on p21-deficient cells is mediated by apoptosis as demonstrated by proteolytic cleavage of PARP assayed by immunoblot (Fig. 1d), by annexin V binding assayed by flow cytometry (Fig. 1e) and by accumulation of sub G0/G1 cells (not shown). The effect of imatinib was partly dependent on p53, as p53-deficient cells were more resistant to high imatinib concentrations (20-60 μM) than parental cells, as shown by cell growth assays (Fig. 1f) and annexin V binding (Fig. 1g).

It was surprising that p21 deficiency resulted in such high sensitivity to the tyrosine kinase inhibitors, as well as its partial dependence on p53, in a similar way than genotoxic drugs as adriamycin or 5-FU. Therefore, we tested the possibility that imatinib indirectly induces DNA damage in HCT116 cells by assaying the accumulation in the cell nuclei of foci with phosphorylated histone H2AX (γ-H2AX, a marker of genomic damage). The results (Fig. 2a) showed that imatinib did not induce nuclear γ-H2AX foci, contrary to cells treated with adriamycin, which were used as positive control. However, imatinib reached its intracellular targets in HCT116, as autophosphorylation of c-Abl kinase was severely inhibited upon treatment with imatinib (Fig. 2b). Interestingly, basal c-Abl expression and activity were significantly higher in HCT116p21$^{-/-}$ than in parental cells. We did not detect expression of other imatinib targets as c-Kit and EGF receptor in HCT116 (not shown). It was striking the higher level of c-Abl activation observed in HCT116p21$^{-/-}$ cells, and we asked whether other kinases besides c-Abl were hyperactivated in HCT116p21$^{-/-}$ cells. Using phospho-specific antibodies we found an enhanced phosphorylation of Akt, Erk and c-Src kinases in HCT116p21$^{-/-}$ cells as compared to parental cells (Fig. 2c). Imatinib also resulted in the inactivation of the c-Src kinase (Fig. 2d), consistent with the described requirement of c-Abl for c-Src activity [34].

3.2 p21 deficiency is not responsible for the hypersensitive phenotype of HCT116p21$^{-/-}$ cells

To directly test whether p21 deficiency is responsible for the hypersensitive phenotype of p21-deficient cells, we generated cell lines with inducible expression of p21. Cells were transfected with an expression vector where the human p21 cDNA is under control of the metallothionein promoter and its expression is induced by Zn^{2+} in the culture media [25]. The kinetics of p21 induction in one selected transfectants (Hp21KO-MTp21) is shown in Fig. 3a. Most of exogenous p21 remains in the cell nuclei, as assessed by immunofluorescence (Fig. 3b). We tested the functionality of the ectopic p21 by assaying the kinase activity of CDK2 upon induction of p21 with Zn^{2+}. The results showed that induction of p21 in Hp21KO-MTp21 resulted in effective inhibition of CDK2 (Fig. 3c). Consistently, induction of p21 reduced the fraction of cells in G1 phase of the cell cycle, as assayed by flow cytometry of PI-stained cells (Fig. 3d), as cells in G1 transverse into G2/M but cannot begin a new S phase. This is consistent with the reported effect of p21 as an inductor of G2 accumulation [35,36].

Having characterized the Hp21KO-MTp21 cell line, we next asked whether restoring p21 expression in HCT116p21$^{-/-}$ cells conferred resistance to imatinib. Surprisingly, the induction of p21 did not alter the sensitivity of the cells towards imatinib, as determined by cell growth (Fig. 3e) and the fraction of apoptotic cells as determined by annexin V binding (Fig. 3f). In addition, induction of p21 in Hp21KO-MTp21 cells did not modify the sensitivity to adriamycin (not shown).

The above results from p21 overexpression suggested that p21 deficiency was not responsible for the enhanced cytotoxic response to drugs. We sought to confirm this by the opposite approach, i.e., decreasing p21 expression in wild-type HCT116 cells through RNA interference. We generated a polyclonal cell line expressing a p21 short-hairpin vector by retroviral transduction. These cells, termed HCT-shp21B, showed reduced levels of p21 protein levels with respect to parental cell line (Fig. 3g). Adriamycin potently up-regulated p21, as previously reported [21,37] and we found that HCT-shp21B showed reduced p21 in the presence of adriamycin, as compared to control cells (Fig. 3g). In contrast, imatinib did not modify p21 levels and the cells also showed reduced p21 levels upon treatment with imatinib, with respect to parental cells (Fig 3g). Thus, we next asked whether p21 suppression modified the cytotoxicity mediated by drugs. We found that the response to adriamycin, 5-fluorouracil, imatinib and gefitinib was similar for HCT116 and HCT-shp21B cell lines (Fig. 3h). Also, HCT-shp21B and parental HCT116 show similar sensitivity to high imatinib concentrations (20-60 μM) (not shown). These results suggest that p21 deficiency is not responsible for the sensitivity to imatinib. In a complementary approach to decrease endogenous p21 we generated cell lines transfected with an conditional Myc, a transcription factor known to repress p21 expression in many cell lines including HCT116 [38,39].We generated HCT116 cells expressing c-MycER, a Myc fusion protein which is activated by 4-hydroxytamoxifen. Addition of 4-hydroxytamoxifen to these cells resulted in p21 down-regulation (Supplemental Fig 1). However, c-Myc activation did not result in a significant change in sensitivity to imatinib or adriamycin, as determined by proliferation assays, annexin V binding and PARP proteolysis (Supplemental Fig. 1). Altogether, the results indicate that p21 deficiency is not responsible for the hypersensitive response of HCT116p21$^{-/-}$ cells to drugs as tyrosine kinase inhibitors or DNA-damaging drugs.

3.3 p53 is stabilized and hyperactivated in HCT116p21$^{-/-}$ cells

In an attempt to explain the higher sensitivity of HCT116p21$^{-/-}$ cells to drugs we analysed the expression of genes related to apoptosis through macroarray hybridization. We found that the mRNA expression of several p53 target genes (e.g., GADD45, DR5, CD95, BAX, CHEK1, TRAF1) was up-regulated in response to imatinib in the p21-deficient cells as compared to parental cells (Fig. 4a). This finding suggested that p53 might be involved in the hypersensitive phenotype of HCT116p21$^{-/-}$. Thus, we studied the status of p53 in HCT116p21$^{-/-}$ and found that p53 protein levels were dramatically elevated with respect to parental cells (Fig. 4b), as already observed [40-43]. Most of the p53 mutations found in cancer render a more stable protein, and it was conceivably that mutation in p53 was selected during the multiple passages of the cell line. However, we did not find mutations in exons 2-11 of the p53 gene of the HCT116p21$^{-/-}$ cell line used in our studies (not shown). In contrast we found that in HCT116p21$^{-/-}$ cells but not in the parental cells, p53 was hyperphosphorylated in residues known to be important for p53 stabilization and/or transactivation: Ser-9, Ser-15 and Ser-392 (Fig. 4b). We did not detect significant changes in the phosphorylation of other residues tested (Ser-6, Ser-20, Ser-37, Ser-46) (not shown). It was expected that these modifications would result in increased p53 stability. To determine p53 stability, HCT116 and HCT116p21$^{-/-}$ cells were treated for the indicated periods of time with 50 μg/ml of the protein synthesis inhibitor cycloheximide and the p53 levels were assessed by immunoblot. The results demonstrated that p53 had higher stability in HCT116p21$^{-/-}$ (Fig. 4c). Moreover, reporter-luciferase assays with three different p53-responsive promoters revealed an increased transcriptional activity of p53 in HCT116p21$^{-/-}$ cells (Fig. 4d). Therefore, HCT116p21$^{-/-}$ expressed higher levels of active p53 than the parental cell line.

We next asked whether the elevated p53 levels in HCT116p21$^{-/-}$ cells depended on p21 deficiency. The results (Fig. 5a) revealed that induction of p21 in Hp21KO-MTp21 did not modify p53 levels and p53 phosphorylation. Also, reduction of p21 levels in HCT116 cells (i.e., in HCT-shp21B cells) did not result in increased total p53 levels or phospho-Ser15-p53 levels (Fig. 5b). Moreover, high transient p21 levels achieved by adenoviral infection in HCT116p21$^{-/-}$ cells did not result in any decrease of p53 levels (Fig. 5c). Cells acutely infected with p21 adenovirus did not show any increase in resistance towards imatinib or adriamycin after 24 h of treatment with respect to cells infected with a control virus, assessed by sub G0/G1 fraction and

annexin V binding (not shown). At longer infection times (36-48h) the p21 virus induced cell death (Fig. 5d). Finally, we could not detect differences in p53 protein levels (as assayed by immunoblot) in ascendant colon from p21-deficient mice [9] and parental mice (not shown). In summary, the results indicate that HCT116p21$^{-/-}$ cells express higher p53 levels and exert higher p53 activity than parental cells, and that this was independent from its p21 deficiency.

3.4 High p53 levels are responsible for the hypersensitive phenotype in HCT116p21$^{-/-}$ cells

We finally asked whether the increased sensitivity of HCT116p21$^{-/-}$ to drugs was a consequence of the p53 overexpression in HCT116p21$^{-/-}$ cells. To test this hypothesis we generated HCT116p21$^{-/-}$ sublines with reduced p53 expression and tested their response to imatinib and adriamycin. Cells were transduced with retrovirus expressing a short-hairpin p53 [27]. The transduced cells (Hp21KO-shp53) expressed reduced p53 levels (Fig. 6a). We also generated transfectant HCT116p21$^{-/-}$ cell lines expressing a p53 fragment (DDp53) that acts as a dominant negative p53 mutant [28]. We chose a cell line termed Hp21KO-DDp53 and the expression of DDp53 was confirmed by immunoblot (Fig. 6b). Cell proliferation assays demonstrated that the cell line with reduced p53 (Hp21KO-shp53) grew faster than the parental HCT116p21$^{-/-}$, and at a similar rate than the wild-type HCT116 cells (Fig 6c).

We next asked whether p53 inactivation in these cells conferred higher resistance to kinase inhibitors. As shown in growth curves of Fig. 7a, silencing of p53 completely rescued the growth inhibition by low imatinib and gefitinib doses, as p21KO-shp53 grew at the same rate than parental HCT116 cells. Confirming our previous results (Fig. 3), the reduction of p21 (i.e., HCT-shp21B cells) did not confer higher sensibility in this assay. The inactivation of p53 with the DDp53 mutant (Hp21KO-DDp53 cells) also reversed the hypersensitive phenotype of HCT116$^{-/-}$ cells to a similar extent than in Hp21KO-shp53 cells (Fig 7b). Moreover, reduction of p53 levels or p53 activity resulted in increased resistance to the apoptosis induced by imatinib, as assessed by annexin V binding assay (Fig. 7c). Proliferation assays also confirmed that impairment of p53 function reverted the hypersensitive phenotype of HCT116p21$^{-/-}$ cells towards adriamycin, imatinib and gefitinib (Fig. 7d). These results are consistent with the increased resistance to high imatinib concentrations of p53-

deficient HCT116, described above (Fig. 1). Thus, reduction of p53 levels or activity rescued the slow-growing and the hypersensitive phenotypes of HCT116p21$^{-/-}$. We showed above (Fig. 2) that despite the hypersenstivity to imatinib, HCT116p21$^{-/-}$ cells had higher c-Abl and c-Src protein kinases activity. Thus we asked whether impairment of p53 function would also antagonize these activities. The results demonstrate that c-Abl and c-Src activities are decreased in Hp21KO-shp53 and Hp21KO-DDp53 with respect to parental HCT116p21$^{-/-}$ cells (Fig. 7e). Taken together, our results strongly suggest that p53 is responsible for the hypersensitivity to tyrosine kinases inhibitors in this model.

4. Discussion

We demonstrate here that HCT116p21$^{-/-}$ cells are dramatically more sensitive to imatinib and gefitinib than parental cells. Both inhibitors are now widely used in therapy of human cancer. Our results extend to tyrosine kinase inhibitors the previous observation with DNA-damaging drugs as adriamycin or 5-FU, which are also more cytotoxic to HCT116p21$^{-/-}$ cells [22,31-33]. However, restoring p21 expression in the p21-deficient cells did not modify the hypersensitive response to imatinib and adriamycin, and silencing p21 in parental cells did not confer higher sensitivity to the drugs. Surprisingly, this hypersensitivity is not due to p21 deficiency but to p53 overexpression in HCT116p21$^{-/-}$ cells, as the silencing or inactivation of p53 confers a lower sensitivity towards these drugs, and similar to that of parental cells.

It is noteworthy and previously unnoticed that p53 also confers sensitivity to gefitinib and imatinib, although ten-fold higher imatinib concentrations are required to achieve a cytotoxic effect in this colon cancer cells than in chronic myeloid leukemia cells (the major clinical application of imatinib). Conversely to HCT116, chronic myeloid leukemia cells express Bcr-Abl kinase as imatinib target. The dependence on p53 for imatinib response in HCT116, which do not express Bcr-Abl but express c-Abl, extends previous findings in chronic myeloid leukemia where p53 inactivation is associated to imatinib resistance [44,45].

p53 overexpression in HCT116p21$^{-/-}$ was due to enhanced stability of the p53 protein. The reason for this effect in HCT116p21$^{-/-}$ cells is unclear, but it is not dependent on p14^{ARF} and Mdm2 expression [42,46] and our unpublished results]. We have shown that HCT116p21$^{-/-}$ cells express high levels of activated c-Abl kinase (Fig. 2b), and it is established that c-Abl stabilizes p53 by inhibiting Mdm2-mediated degradation of p53 [reviewed in [47]. So, high c-Abl expression could explain the stability and elevated p53 levels in HCT116p21$^{-/-}$ cells. It is also noteworthy the high levels of activated kinases (e.g. c-Abl, Akt, c-Src and Erk2) in p21-deficient cells. This is striking as some of these kinases have been associated to cell survival in different systems. We hypothesize that the HCT116p21$^{-/-}$ cell line, besides its deficiency in p21, acquired additional genetic or epigenetic changes during the selection process leading to high basal kinase activity which would stabilize p53 through different pathways. This would result in cells showing a slower growth rate and higher sensitivity to drugs.

Regardless of the mechanism that led to the high-p53 and hypersensitive phenotype of HCT116p21$^{-/-}$ cells, our results invite to reconsider many of the reported

conclusions on the anti-apoptotic effect of p21 in colon cancer cells. An antiapoptotic role for p21 has been demonstrated in several cell culture models from different tissues [reviewed in [7,8,48], and can explain the decreased rate of radiation-induced tumorigenesis p21-deficient mice [12,49]. In the HCT116 model, comparative work with HCT116 and HCT116p21$^{-/-}$ cell lines has generated multiple reports arguing that p21 loss sensitizes these cells for apoptosis induced by a number of antitumoral drugs (Supplemental Table 1). Although it is unknown whether our results can be extended to all drugs, the work described here challenge these conclusions as we demonstrate that the hypersensitive phenotype for the drugs tested in our work, depends on the high p53 levels in the HCT116p21$^{-/-}$ cell line and not on the p21 deficiency.

In support of this hypothesis, a recent report indicates that the hypersensitivity to chromium-induced DNA damage in HCT116p21$^{-/-}$ is also mediated by p53 [50]. *In vivo* inactivation of p21 in mice resulted in a significant increase in the frequency and size of intestinal tumors in two different models of colon carcinogenesis [51,52]. These results are not consistent with the idea that p21 deficiency confers an apoptosis-prone and slow-growth phenotype whereas they are in agreement with our results in the HCT116 model. Moreover, trials in human colorectal cancer with different drugs have failed to demonstrate a favorable prognostic role for elevated p21 expression in overall survival [reviewed in [53]. This observation is at odds with an anti-apoptotic function of p21 in human colon cancer cells. We conclude that p21 does not have a protective effect on drug-induced cell death in the HCT116 model and that the role of p21 in colon cancer cell apoptosis should be re-evaluated.

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Figure legends

Fig. 1. HCT116p21$^{-/-}$ cells are hyper-sensitive to the cytotoxic effects of imatinib. (**a**) Cells of the indicated cell lines were treated with the indicated concentrations of imatinib, gefitnib, adriamycin and 5-FU. Cell proliferation was determined after 48 h and expressed as a percentage of the proliferation of untreated cells. (**b**) Clonogenic assay of the indicated cell lines treated with imatinib, gefitinib and 5-FU for 5 days after crystal violet staining. (**c**) Growth curves of the indicated cell lines in the presence of imatinib. "W" indicates the time point at which the cells were washed to remove imatinib. Note that the scales for both cell lines are different. Dotted line in the left graph corresponds to untreated HCT116p21$^{-/-}$ for comparison with wild-type HCT116. (**d**) Apoptosis assessed by the proteolytic cleavage of PARP. HCT116 and HCT116p21$^{-/-}$ cells were treated with the indicated concentrations of imatinib and 0.5 μM adriamycin (ADR) for 48 h. Whole cell protein extracts were analysed by immunoblot with antibodies anti-PARP and anti- α-tubulin as a loading control. (**e**) Apoptosis induction by imatinib as assessed by annexin V binding. Cells were treated with different imatinib concentrations for 48 h. Annexin V binding was determined by flow cytometry. (**f**) Cell proliferation of HCT116 and HCT116p53$^{-/-}$ cells treated with high imatinib concentrations for 48 h. Data are means from three experiments ± S.E.M. (**g**) Apoptosis induction by imatinib as assessed by annexin V binding in cells treated with high imatinib concentrations for 48 h. Data are means from two experiments ± S.E.M.

Fig. 2. Imatinib targets in HCT116p21$^{-/-}$. (**a**) Imatinib does not induce H2AX phosphorylation. Cells were treated with imatinib and (as a positive control) with 0.5 μM adriamycin for 48 h and fixed with paraformaldehyde. The presence of phosphorylated H2AX histone (γ-H2AX) was detected by immunofluorescence. The cells were counterstained with DAPI to stain nuclei and the fraction of positive cells is indicated in each case. Representative images are shown. (**b**) c-Abl inactivation by imatinib in HCT116 and HCT116p21$^{-/-}$ cells. Cells were incubated for 48 h with imatinib and c-Abl was determined by immunoblot. To determine the phosphorylation of c-Abl the blot was probed with an anti-phospho-tyrosine antibody. Actin levels were also determined to assess protein loading. (**c**) Protein kinase activation in HCT116p21$^{-/-}$. Cells were lysed and the levels of kinases indicated at the right were determined by immunoblot. Arrow marks the Erk2 band. Actin levels were also determined as a loading control. (**d**) c-Src inactivation by imatinib in HCT116 and HCT116p21$^{-/-}$ cells. Cells were incubated for 48 h with imatinib and phospho-c-Src and c-Src were determined by immunoblot.

Fig. 3. Ectopic expression of p21 does not rescue the hypersensitive phenotype of HCT116p21$^{-/-}$.(**a**) Induction of p21 in Hp21KO-MTp21cells. Cells were incubated with 75 μM $ZnSO_4$ for the indicated periods of time and protein extracts were analysed by immunoblot with anti-p21 and anti-α-tubulin antibodies. (**b**) Induction and nuclear localization of p21 in Hp21KO-MTp21 cells. Immunofluorescence analysis with anti-p21 antibody was performed in cells treated for 12 h with 50 μM $ZnSO_4$. Cells were counterstained with DAPI to stain nuclei. (**c**) Induction of p21 in Hp21KO-MTcells inhibits activity kinase of CDK2. Cells were incubated with 75μM $ZnSO_4$ during 24 hours. Lysates were immunoprecipitated with anti-CDK2 antibodies and assayed for kinase activity using histone H1 as substrate. Levels of CDK2 and p21 in the immunoprecipitates were determined by immunoblot. (**d**) Cell cycle analysis of HCT116, HCT116p21$^{-/-}$ and Hp21KO-MTp21 cells treated with 75 μM $ZnSO_4$ for 4 days and assayed for the PI incorporation by flow cytometry. (**e**) Micrographs of HKO-MTp21 cells treated with 20 μM imatinib for 48 h in the presence and absence of 75 μM $ZnSO_4$ showing similar sensitivity in the presence and absence of the p21 inducer. (**f**) Apoptosis induction by imatinib as assessed by Annexin V binding. Cells of the indicated cell lines were treated with 20 μM imatinib for 24 h. Annexin V binding was determined as in Figure 1. (**g**) Silencing of p21. Lysates were prepared from parental HCT116 and derived cell lines expressing two different short-hairpin constructs for human p21, one competent for silencing (HCT-shp21B) and another used as a negative control (HCT-shp21C), as well as cells transduced with empty vector (HCT-pRS). Cells were treated for 16 h with adriamycin (upper panel) and with imatinib (lower panel) to show p21 levels in the presence of the drugs. p21, α-tubulin and actin were detected by immunoblot. A short exposure of the film is also shown to demonstrate p21 downregulation in the presence of adriamycin. HCT116p53$^{-/-}$ cells was used as negative control of p53 expression in the lower-panel. (**h)** Proliferation assay of the indicated cell lines incubated with increased concentrations of adriamycin, 5-fluorouracil, gefitinib and imatinib. The cells were stained with crystal violet after 48 h of treatment. The numbers in the wells represent densitometric units normalized to the value of untreated cells.

Fig. 4. p53 is stabilized and hyperactivated in HCT116p21$^{-/-}$ cells. (**a**) Induction of p53-target genes in HCT116p21$^{-/-}$ treated with imatinib. HCT116p21$^{-/-}$ and HCT116 were treated with 15 μM imatinib for 48 h. The RNA was prepared and labelled and the expression of p53 target genes was determined by macroarray hybridization as described in Material and Methods. Data are normalized to the expression of each gene in parental HCT116 cells. (**b**) Levels of

phosphorylated p53 in HCT116 and HCT116p21$^{-/-}$ cells treated with imatinib for 48 h. Cells were lysed and the immunoblot were prepared using phospho-specific antibodies. As a control, a lysate from HCT116 cells treated with 0.5 μM adriamycin for 48 h (HCT+Adr) was included. (**c**) Immunoblot of HCT116 and HCT116p21$^{-/-}$ cells treated for the indicated periods of time with 50 μg/ml of cycloheximide (Chx). The blot was probed with anti-p53 and anti-actin antibodies. (**d**) p53 transcriptional activity of HCT116 and HCT116p21$^{-/-}$ measured with the three indicated luciferase constructs carrying p53-responsive elements. Data are means of two different experiments done in duplicate and are normalized to the values in HCT116 cells. Error bars represent S.E.M.

Fig. 5. Induction or repression of p21 does not modify p53 expression in HCT116 cells. (**a**) Induction of p21 does not modify p53 expression in HCT116. Whole-cell lysates from Hp21KO-MTp21 cells grown in the absence or presence of 50 μM $ZnSO_4$ for 24 h (to induce p21) were analysed by immunoblot with antibodies against p53, p21, actin and Ser-15- and Ser-392-phosphorylated p53. (**b**) Silencing of p21 does not modify p53 expression in HCT116 cells. Lysates of HCT1-shp21B (where p21 is silenced, Figure 3d), HCT-shp21C (expressing a control siRNA) and HCT-pRS (transduced with the empty virus) were analysed by immunoblot with the antibodies anti-p21, anti-total p53 and anti-phospho-serine-15-p53. The filter was stained with Ponceau Red to assess protein loading (**c**) Overexpression of p21 by adenoviral infection does not modify p53 expression in HCT116p21$^{-/-}$ cells. Cells were infected with adenovirus carrying p21 gene and GFP [26] and 24 h after infection whole-cell protein extracts were analysed by immunoblot with antibodies against p21, p53 and α-tubulin. (**d**) Overexpression of p21 did not confer protection to infected cells. HCT116p21$^{-/-}$ were infected with adenovirus p21 and GFP and 6h after infection cells were treated with 20 μM imatinib and 1 μM adriamycin and micrographs were taken after 30 h of treatment.

Fig. 6. Impairment of p53 activity in HCT116p21$^{-/-}$ cells rescues the slow-growing phenotype. (**a**) Silencing of p53 in Hp21KO-shp53 cells. Cell lysates from the indicated cell lines were analysed by immunoblot with anti-p53. The filter was stained with Ponceau Red to assess protein loading. (**b**) Expression of DDp53. Cell lysates from the indicated cell lines were analysed by immunoblot with anti-p53 antibody. The filter was stained with Ponceau Red to assess protein loading (**c**) Cell proliferation of HCT116, HCT116p21$^{-/-}$ and Hp21KO-shp53 cells. Cells were plated at equivalent densities in parallel dishes and tripsinized and counted during four days. Data are mean from two experiments ± S.E.M.

Fig. 7. Hypersensitivity to imatinib of HCT116p21$^{-/-}$ cells depends on high p53 levels and activity. (**a**) Growth curves of the indicated cell lines in the presence of imatinib and gefitinib. Cells were plated at equivalent densities and tripsinized and counted during five days. (**b**) Micrographs of the indicated cell lines treated with 20 μM imatinib for 48 h showing the reduced sensitivity to imatinib of Hp21KO-shp53 and Hp21KO-DDp53. (**c**) Cells were treated for 48 h with imatinib and the fraction of cells binding annexin V was determined as in Figure 1. (**d**) Proliferation assay of the indicated cell lines incubated with increased concentrations of adriamycin, imatinib and gefitinib. The cells were stained with crystal violet after 48 h of treatment. The numbers in the wells represent densitometric units normalized to the growth of untreated cells. (**e**) Silencing of p53 protein in HCT116p21$^{-/-}$ downregulated the activity of c-Src and c-Abl. Whole-cell lysates from HCT116, HCT116p53$^{-/-}$, HCT116p21$^{-/-}$, Hp21KO-shp53, Hp21KO-DDp53 were analysed by immunoblot with antibodies against p53, p-c-Src, c-Src, p-Abl, c-Abl and actin.

Figure

Figure 1

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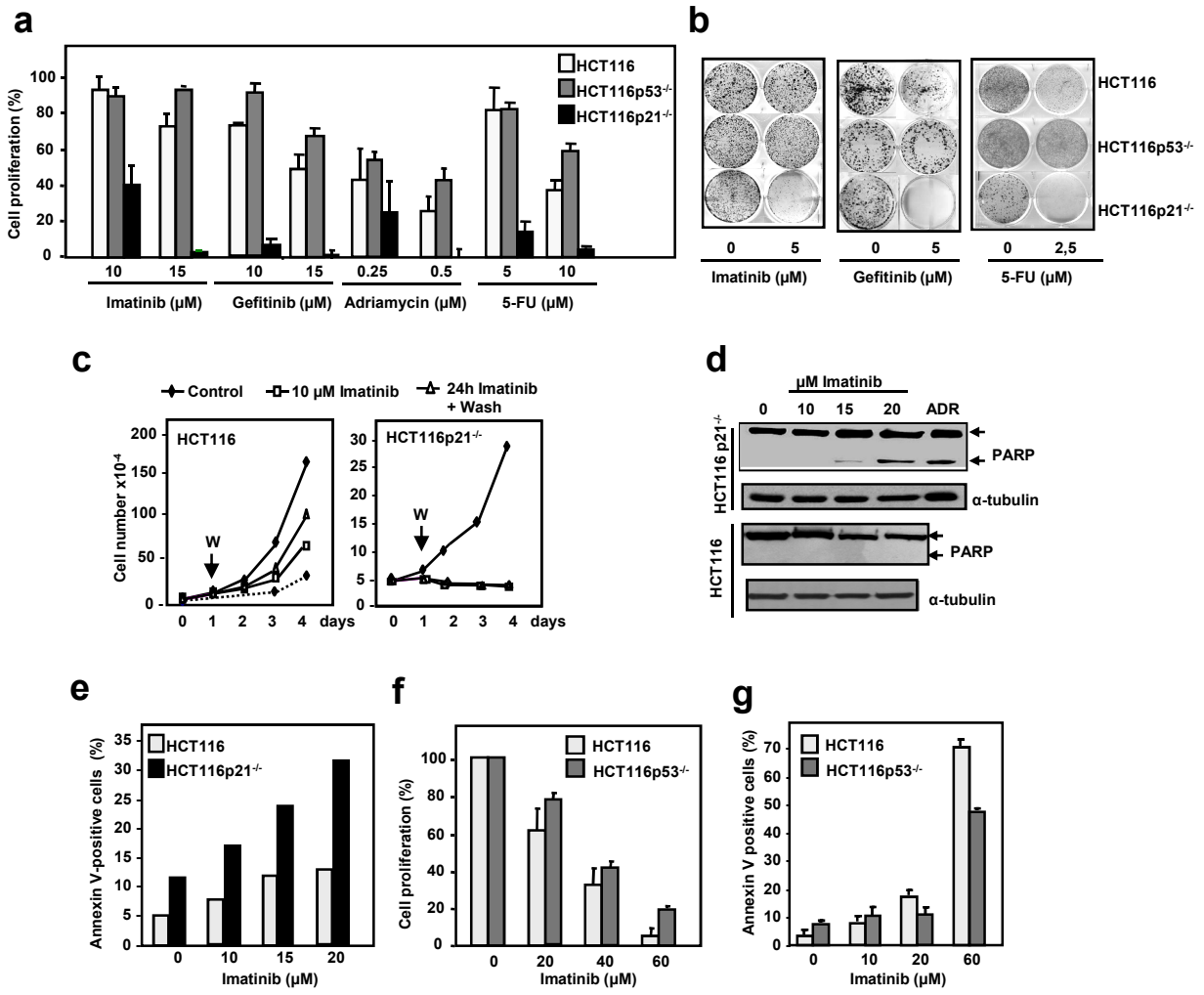

Figure

Figure 2

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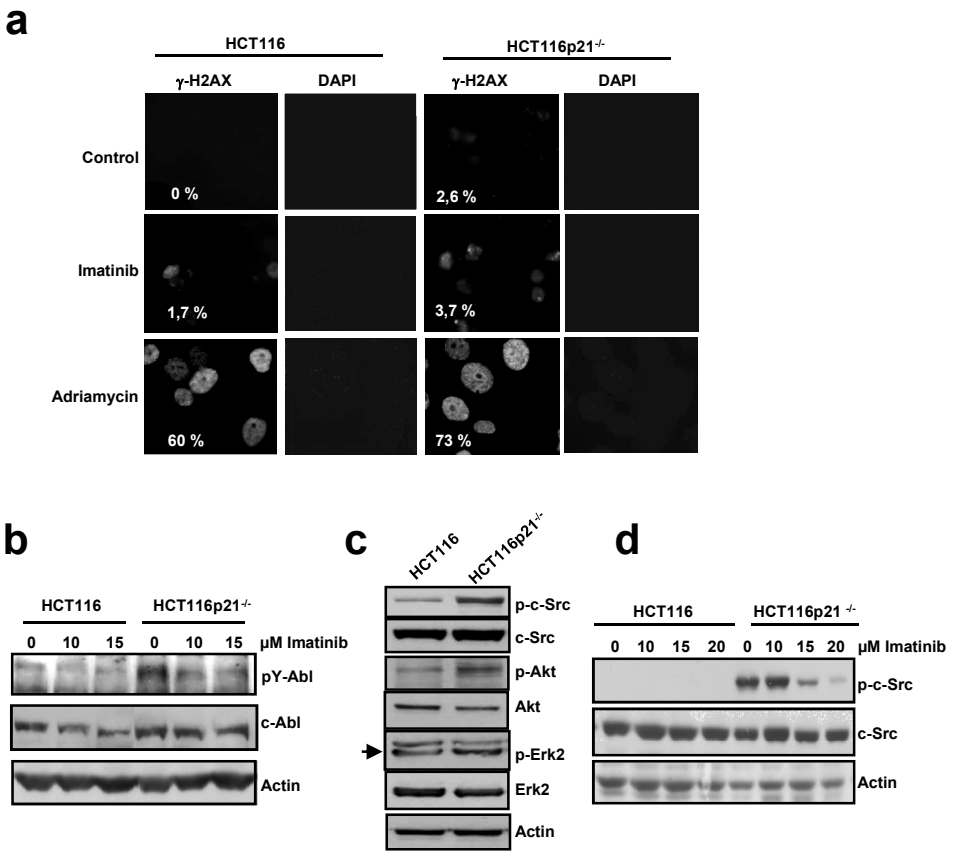

Figure 3

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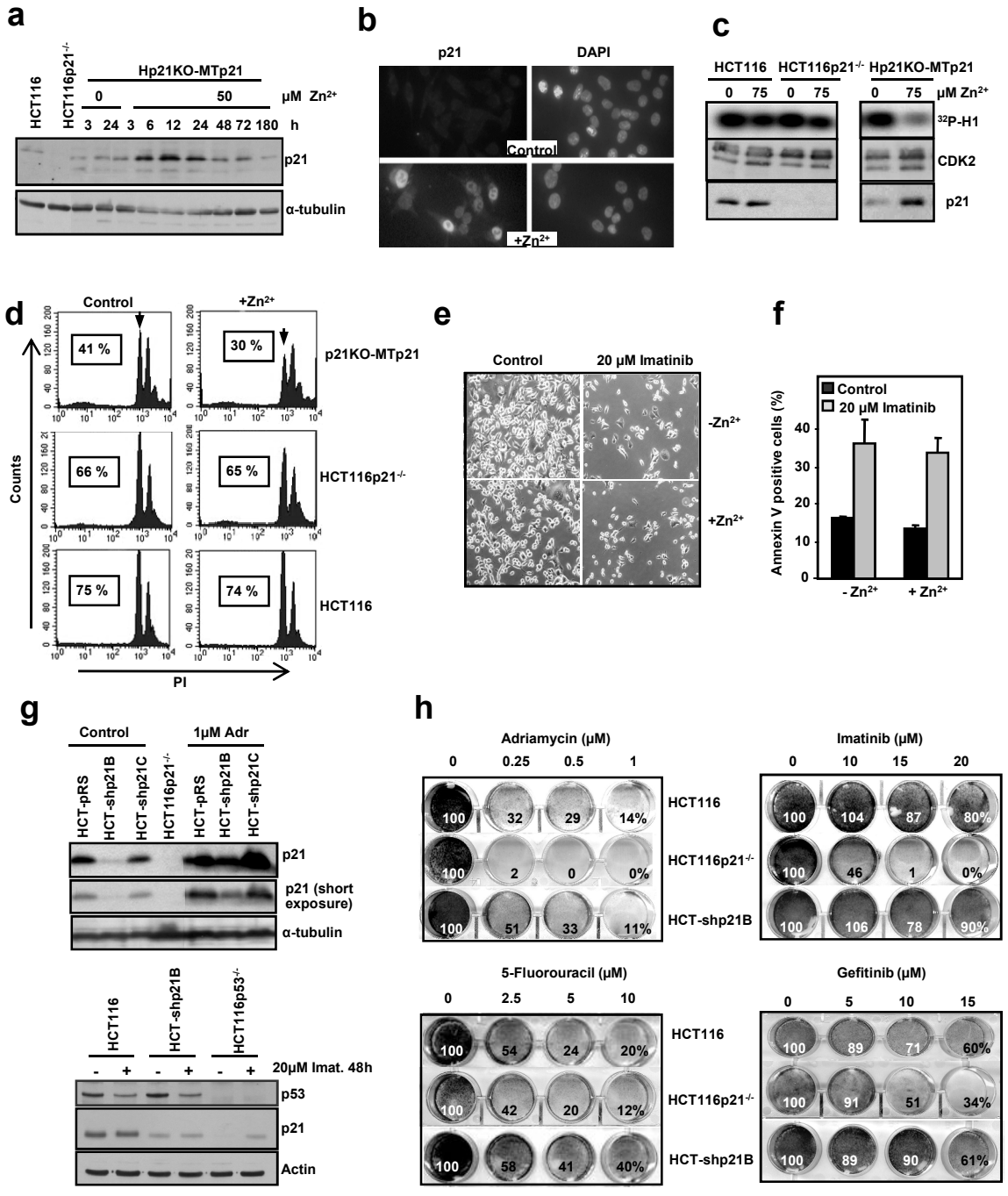

Figure 4

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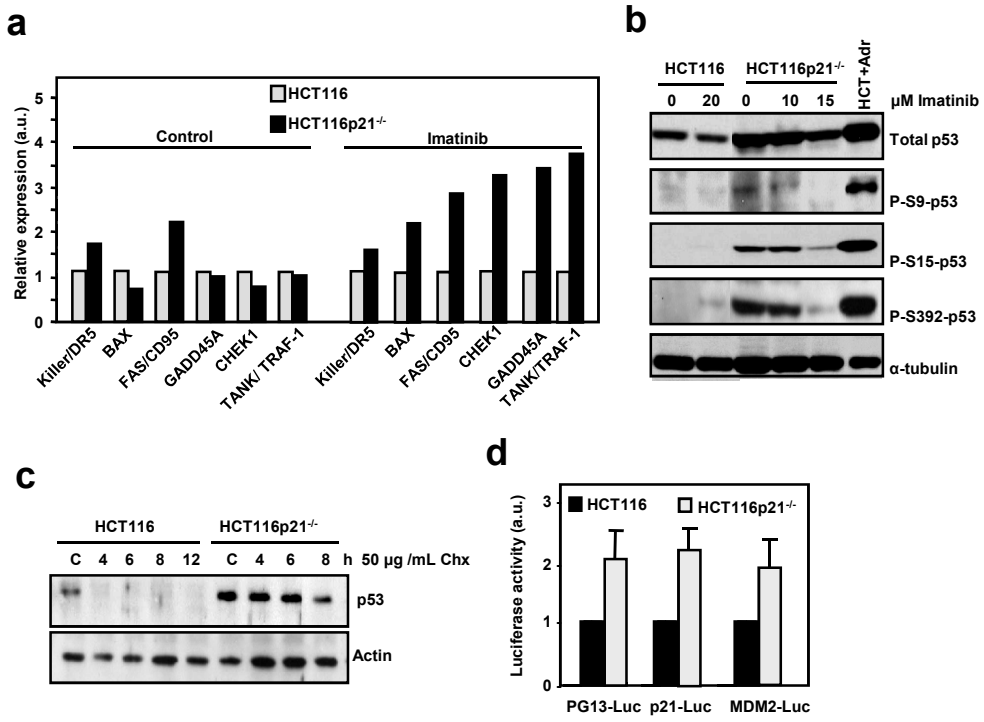

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Figure 5

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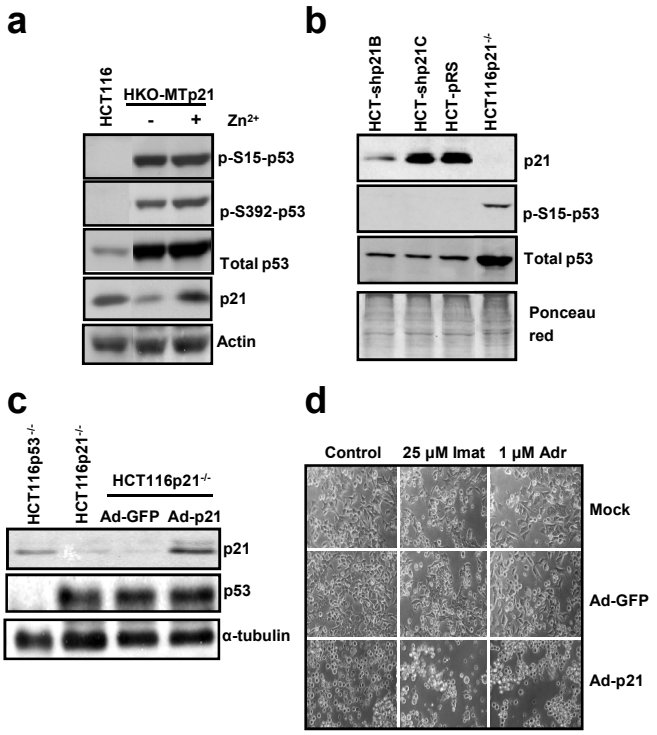

Figure

Figure 6

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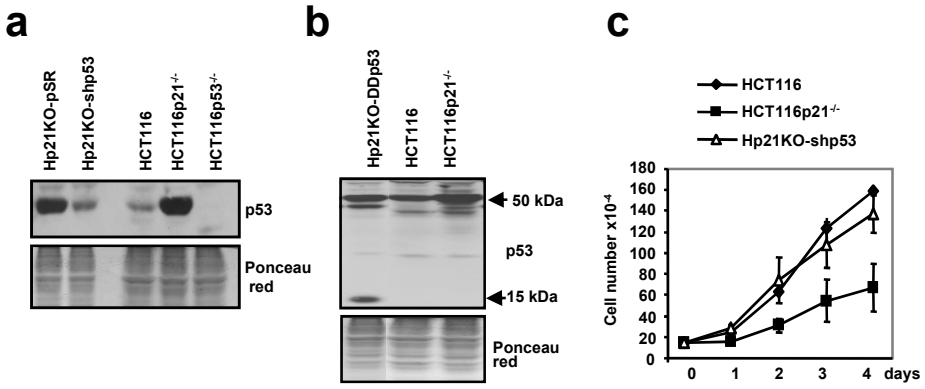

Figure 7

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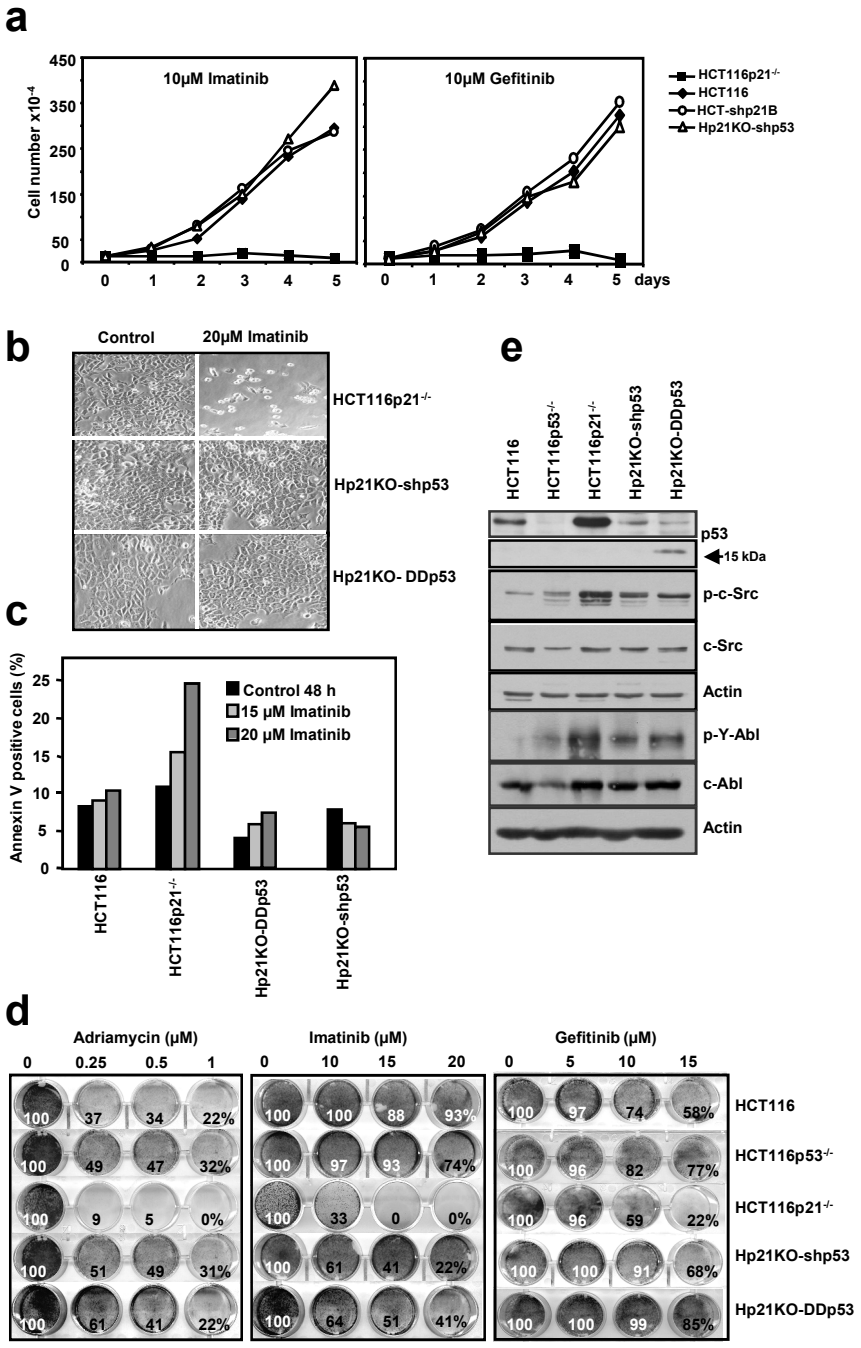

Supplementary Material

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HCT116 cells deficient in p21^{Waf1} are hypersensitive to tyrosine kinase inhibitors and adriamycin through a mechanism unrelated to p21 and dependent on p53

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COVER LETER

Dear *DNA Repair* Editor

We thank you very much for the review of our manuscript (MS) entitled "HCT116 cells deficient in p21Waf1 are hypersensitive to tyrosine kinase inhibitors and adriamycin through a mechanism unrelated to p21 and dependent on p53" by Ferrandiz et al.

The comments of the reviewers have certainly improved the MS. After performing a series of new experiments we believe that we have appropriately responded to all the points raised by the two reviewers. To illustrate of our responds we include in our response letter four "Figures for Reviewers". Also, as the request of the reviewers we have included some new data in the MS, which contains now 7 figures so as to accommodate these new data.

The manuscript contains seven figures plus a supplementary Table and a supplementary figure.

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The point-by-point response to the reviewer's comments follow.

Reviewer #1

1.HCT116 p21-/- cells seem to have higher levels of phosphorylated c-Abl and c-Src than parental HCT116 cells (Fig. 2). Could this contribute to the greater sensitivity of HCT116 p21-/- cells to TKIs?

The western blot of Fig 2 has been repeated with cell lysates from three independent experiments, with the same results, i.e., an increased level of Phospho-c-Abl and Phospho-c-Src in HCT116 $p21^{-/-}$ cells. The possibility that higher levels of phospho-c-Abl contribute to the greater sensitivity to the drug is not consistent with the fact that HCT116 $p21^{-/-}$ are hypersensitive to a c-Abl inhibitor as imatinib. To test the point raised by the reviewer about c-Src, we have treated the cells with the c-Src inhibitor SU6656. We found that c-Src inhibition (assessed by p-c-Src levels) did not result in increased sensitivity of HCT116 cells and did not increase the sensitivity of imatinib (Fig. 1 for reviewers).

2. To conclude that the role of p21 on apoptosis of HCT116 cells should be re-evaluated, the authors need to perform more studies with genotoxic agents. They have examined the response to adriamycin and 5-FU, but in many cases the analysis is not complete. For example, in Fig. 3h, the panel for HCT116 p21-/- cells treated with 5-FU is missing. Fig. 1 shows the response of cells to 5-FU only in panel A, etc.

We have performed a series of experiments with gefitinib and 5-FU. In the Version 2 of the MS we present these data in the new figures 1b, 3h and 7a. The results fully confirm those already shown for adriamycin and imatinib, i.e., a higher sensitivity of HCT116 $p21^{-/-}$ cells towards these drugs.

3. The experiment showing that restoring expression of p21 does not rescue the sensitivity ot imatinib needs to be improved, because only a small fraction of the cells express p21 after addition of Zn (Fig. 3b). Alternatively, the authors could use adenoviral infection (see comment 6, below).

All Hp21KO-MTp21 cells show p21 induction after $ZnSO_4$ addition, as assessed by immunofluorescence and comparing with controls without Zn^{2+}. However, some cells are particularly bright, and in the image presented in the first version of the MS the settings of brightnees/contrast gave the false idea that there are cells not expressing p21. In the Figure 3b of the MS-version 2, which we present again the image with a brighter setting so as all cells in the filed can be observed. We also show several more fields from an independent experiment in the Figure 2 for Reviewers. Importantly, we have used in all these images the same bright/contrast settings for the uninduced cells (i.e., without Zn^{2+}) and for the induced cells. Although essentially all Hp21KO-MTp21

cells overexpress p21 in response to the inducer, we do not know why there are cells expressing more p21 than others. It also happens with other cell types as leukemia cells with Zn-inducible p21 (Muñoz et al., J Biol. Chem., 2005) and p27 (Acosta et al., Mol. Cell Biol. In press). We hypothesize that there is a cell-cycle dependent transient stabilization of these CKIs.

4. The experiment showing p21 levels in HCT116 cells depleted of p21 after adriamycin treatment is very interesting (Fig. 3g). Apparently, adriamycin induces a significant increase in p21 levels, such that now in the adriamycin-treated p21-depleted cells the levels of p21 are similar to the untreated non-p21-depleted cells (lanes 6 versus 1). Thus, there is plenty of p21 in the p21-depleted cells and this creates a problem in interpretation. Can the authors show the levels of p21 in the imatinib-treated cells? Can they achieve better suppression of p21 expression?

The experiments of Fig 3g were performed only to demonstrate the reduced p21 levels in the HCT-shp21B cell line. The results show that the expression of p21 was efficiently down-regulated in the absence of drugs. As we know that adriamycin is a potent p21 inducer we also checked p21 levels in the presence of the drug (right lanes of the figure). The results show that despite this induction, the levels of p21 in adriamycin-treated HCT-shp21B cells is two-fold less (as assessed by film densitometries in Fig. 3g) than in adriamycin-treated control HCT116 control cells. Despite of that, we could not detect any significant differences between both cell lines in the sensitivity to the four drugs tested: adriamycin, imatinib, gefitinib and 5-fuorouracil. Moreover we have addressed the point raised by the reviewer and determined the p21 levels in both cell lines upon imatinib treatment. The results (shown the Fig 3g bottom panel of the MS-Version 2) show that, conversely to adriamycin, imatinib does not induce p21. The figure again shows the reduced p21 levels in HCT-shp21B cells.

5. The high level of p53 activity in the HCT116 p21-/- cells (Fig. 4) could be due to the oncogenic signal provided by the high levels of phosphorylated c-Abl and c-Src in these cells. This could also explain why imatinib suppresses p53 activation (Fig. 4b).

As indicated in the response to point #1, high c-Abl activity cannot be the reason fr the hypersentivity to imatinib. We also addressed the reviewer's point by testing the effect of the c-Src inhibitor SU6656 on p53 levels. We found that upon c-Src inhibition (assessed by Phospho-Src immunoblot) augments the p53 levels in parental cells (not shown), but does not modify the p53 levels HCT116p21$^{-/-}$ cells (Fig. 1 for Reviewers). Furthermore, we think that unambiguously demonstrate that p53 overexpression is responsible for the high-sensitivity phenotype of HCT116p21$^{-/-}$ cells. First we show that p53 reduction or inactivation rescues the low-proliferation phenotype of HCT116p21$^{-/-}$ cells. We have repeated these experiments and now are presented in the Fig. 6c of the MS-Version 2 with error bars. Moreover, we present new data showing that silencing or inactivation of p53 efficiently antagonizes this hypersensitivity towards all tested drugs (Fig 7 of MS-Version 2). Finally we also present new data showing that p53 down-regulation (Hp21KO-shp53) or inactivation (Hp21KO-DDp53) results in a decrease in phosphorylated c-Abl and c-Src activity (new Fig. 7e of MS-Version 2). These results demonstrate that the hyperactivation of these kinases depend on the high p53 levels in the HCT116p21$^{-/-}$ cells.

6. Did the authors examine the sensitivity of adeno-p21-infected cells (Fig. 5c) to imatinib, 5-FU, etc?

We have performed the experiments suggested by the reviewer. The adenovirus infection was demonstrated by immunoblot of lysates prepared 24 h after the infection

(Figure 3a for Reviewers). 24 h after imatinib treatment we did not observe any protective effect of p21 (Fig. 3b for referees) At longer infection times (36-48 h) the p21 virus induced cell death (new Fig 5d of MS-version2).

Reviewer #2:

1. The title wording, "..hypersensitive to tyrosine kinase inhibitors.through a mechanism unrelated to p21 and dependent on p53," implies that a p53-dependent mechanism of HCT116p21-/- cell sensitivity to multiple tyrosine kinase inhibitors was demonstrated. Given the data in the manuscript, the authors can make this claim for imatinib but not gefitinib. While the gefitinib sensitivity of HCT116p21-/- cells is not due to p21 deficiency (Fig. 3h), the p53-dependence of this sensitivity was not shown for gefitinib. The authors should show (or at least cite as "data not shown" if this experiment was done) an increase in gefitinib resistance in Hp21KO-shp53 and Hp21KO-DDp53 as was done for imatinib and adriamycin (Fig. 6f).

The reviewer is right in this criticism. We have performed new experiments comparing the sensitivity of HCT116p21$^{-/-}$, Hp21KO-shp53, Hp21KO-DDp53 and parental HCT116 towards adriamycin, gefitinb, imatinib and 5-FU (previously only shown for adriamycin and imatinib). The data shows that silencing of p53 with the siRNA-producing construct or inactivation with the dominant negative construct completely abolishes the hypersensitive phenotype as measured by cell proliferation/viability curves. This is presented in the Fig 7 of the MS-version 2. Moreover, p53 inactivation rescues the slow growing phenotype of HCTp21KO cells (Fig 7) These data, together with the data already presented in the Fig. 6 of the first version of the MS, reinforces our hypothesis

2. The word "that" should be "than" in the sentence (1st paragraph of results, pg.6) ".these cells were more resistant to the kinase inhibitors that the p21-deficient ."

The sentence has been changed.

3. A period is needed after the last sentence on pg. 6.

The period has been inserted

4. Consider changing the sentence (1st sentence, pg 7) ".as well as its partial dependence on p53, in a similar way than genotoxic drugs as adriamycin or 5-FU." A suggested version is, ".as well as its partial dependence on p53, in a similar way as the genotoxic drugs adriamycin and 5-FU."

The reviewer is right and the sentence has been changed.

5. Hp21KO-MTp21 cells without zinc addition show a decrease in G1 population compared to HCT116p21-/- cells. Also, the decrease in the G1 population with zinc addition is modest. This needs to be discussed.

HCT21KO and HCTp21KO-MTp21 (without zinc) grow slower that parental cells with extended cell cycles, thus showing a higher G2 fraction. The experiments of Fig 3d were performed to show that p21 induction with zinc produced the described G1 to G2 shift. We now give references to support this p21 effect (Refs 35 and 36 in the MS-Version 2)

6. The correct name for p21 is Cdkna1 or CDKNA1 for the human gene, so I would change the sentence on p.2 to "Cdkna1/p21Waf1/p21Cip1" (p21 herein after) is a member.

The reviewer is right and the sentence has been changed.

7. The authors state that "Finally, we could not detect differences in p53 proteinlevels (as assayed by immunoblot) in ascendant colon from p21-deficient mice and parental mice (not shown). In summary, the results indicate that HCT116p21-/- cells express higher p53 levels and exert higher p53 activity than parental cells, and that this was independent from its p21 deficiency." The authors are to be commended for doing these controls but this raises the issue of the generality of the effect: e.g. is it specific to tumor cells or perhaps only Hct116? Considering how frequently this line is used, it's worth reporting their observations.

We show the data in the Fig. 4 for Reviewers. The mice are from Jackson Labortories (B6;129S2-*Cdkn1a*^{tm1Tyj}/J) and described in Brugarolas et al., Nature 377(6549):552-7, 1995. All this information were lacking and now we provide it the Methods section of the MS-Version 2

8. The p21-/- phenotype was reversed by inhibition of p53 as shown in Fig. 6. Regarding elevation of of phospho-Src, phospho-Abl, and others in Fig. 2, were these reversed by inhibition of p53? This would provide some mechanistic info for their observation

First, we show in the new version of the MS that p53 reduction or inactivation rescues the low-proliferation phenotype of HCT116p21$^{-/-}$ cells (Fig. 6c), and that silencing or inactivation of p53 efficiently antagonizes this hypersensitivity towards all tested drugs (Fig 7 of MS-Version 2). Finally we also present new data showing that p53 down-regulation (Hp21KO-shp53) or inactivation (Hp21KO-DDp53) results in a decrease in phosphorylated c-Abl and c-Src activity. The data are now shown in the Fig. 7e of MS-Version 2. This result demonstrates that the hyperactivation of these kinases depend on the high p53 levels in the HCT116p21$^{-/-}$ cells.

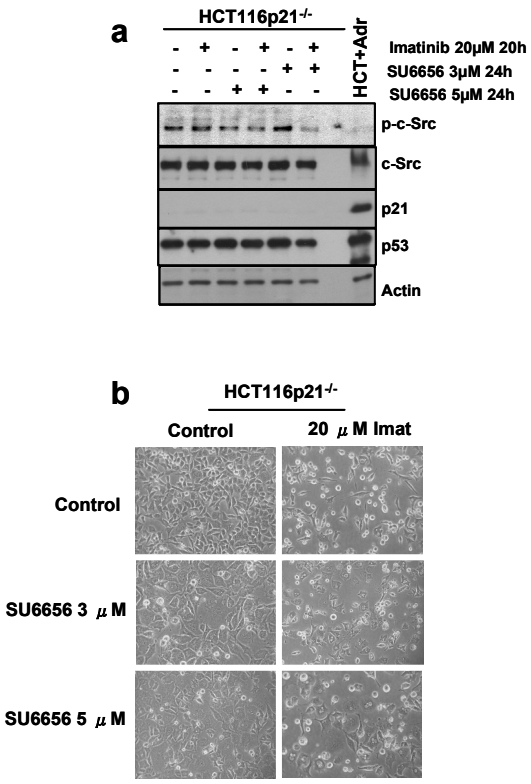


Fig. 1. c-Src inhibition does not modify the sensitivity of imatinib. (**a**) HCT116p21$^{-/-}$ cells were treated with the c-Src inhibitor SU6656 for 24h and/or imatinib for 20h and p-c-Src, c-Src, p21, p53 and actin were analysed by immunoblot. As a control, a lysate from HCT116 cells treated with 0.5 μM adriamycin for 48 h (HCT+Adr) was included.
(**b**) Micrographs of HCT116p21$^{-/-}$ cells treated with SU6656 for 48h and 20 μM imatinib showing similar sensitivity of imatinib in the presence and absence of the c-Src inhibitor.

Figure 2 for referees Ferrandiz et al.

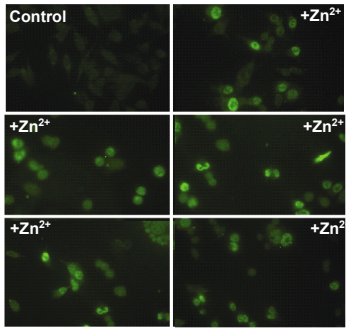
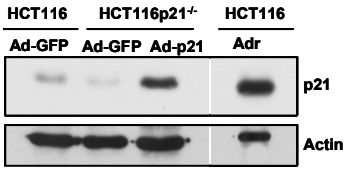


Fig. 2. Induction and nuclear localization of p21 in Hp21KO-MTp21 cells. Immunofluorescence analysis with anti-p21 antibody was performed in cells untreated (Control) or treated for 12 h with 50 μ M $ZnSO_4$ (Zn^{2+}).

Figure 3 for referees Ferrandiz et al.

a



b

	Sub-G0/G1		Annexin V	
	control	**Imatinib**	**control**	**Imatinib**
HCT116-AdGFP	8,8%	11,5%	5,3%	9,1%
HCTp21$^{-/-}$ AdGFP	10,2%	18,4%	7,9%	8,6%
HCTp21$^{-/-}$ Adp21	16,4%	25,6%	11,7%	12,6%

Fig. 3. HCT116p21$^{-/-}$ cells infected with p21 adenovirus were sensitive to imatinib. (**a**) Cells were infected with adenovirus carrying p21 gene and GFP and 24 h after infection whole-cell protein extracts were analysed by immunoblot with antibodies against p21 and actin. As a control, a lysate from HCT116 cells treated with 0.5 μM adriamycin was included. (**b**) Apoptosis induction after treatment with imatinib for 24 h. Fraction of cells in sub G0/G1 phase and annexin V binding were determined by flow cytometry.

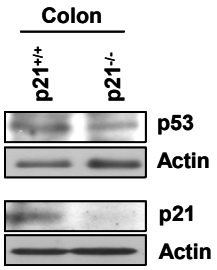


Fig. 4. Colon samples from parental mice and p21-deficient mice do not show differences in p53 protein levels. Whole-cell lysates from ascendant colon tissues were analysed by immunoblot with antibodies against p21, p53 and Actin. A representative blot is shown.

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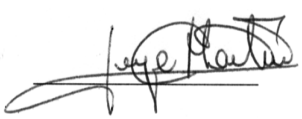

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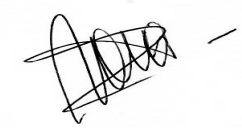

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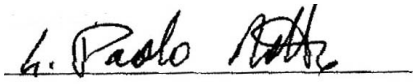

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