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Report

Mutant p53 protein localized in the cytoplasm inhibits autophagy

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Abbreviations: BH3, Bcl-2 homology domain 3; DAPK1, death-associated protein kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; FBS, fetal bovine serum; NES, nuclear export signal; NLS, nuclear localization sequence; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; PVDF, polyvinylidene difluoride; RNAi, RNA interference; SEM, standard error of the mean; TBS, TRIS-buffered saline; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; WT, wild type

Key words: Bcl-2, cancer, GFP-LC3, human colon carcinoma HCT 116 cells, MDM2, p53 hot-spot mutations

The knockout, knockdown or chemical inhibition of p53 stimulates autophagy. Moreover, autophagy-inducing stimuli such as nutrient depletion, rapamycin or lithium cause the depletion of cytoplasmic p53, which in turn is required for the induction of autophagy. Here, we show that retransfection of *p53*$^{-/-}$ HCT 116 colon carcinoma cells with wild type p53 decreases autophagy down to baseline levels. Surprisingly, one third among a panel of 22 cancer-associated p53 single amino acid mutants also inhibited autophagy when transfected into *p53*$^{-/-}$ cells. Those variants of p53 that preferentially localize to the cytoplasm effectively repressed autophagy, whereas p53 mutants that display a prominently nuclear distribution failed to inhibit autophagy. The investigation of a series of deletion mutants revealed that removal of the DNA-binding domain from p53 fails to interfere with its role in the regulation of autophagy. Altogether, these results identify the cytoplasmic localization of p53 as the most important feature for p53-mediated autophagy inhibition. Moreover, the structural requirements for the two biological activities of extranuclear p53, namely induction of apoptosis and inhibition of autophagy, are manifestly different.

Introduction

Autophagy has recently been recognized as one of the pivotal responses to cellular stress. During macroautophagy (hereafter referred to as autophagy) portions of the cytoplasm and intracellular organelles are sequestered within characteristic double- or multi-membraned vacuoles (autophagosomes) and finally delivered to lysosomes for bulk degradation.[1-3] Thanks to autophagy, cells can adapt their metabolism to conditions of nutrient deprivation or hypoxia. Autophagy allows indeed for the controlled catabolism of cellular macromolecules, and therefore generates new metabolic substrates that contribute to the maintenance of bioenergetic and biosynthetic homeostasis. Beyond this role in adaptation to stress and/or adverse metabolic conditions, autophagy constitutes an essential process to eliminate intracellular pathogens as well as potentially toxic aggregate-prone proteins and damaged cytoplasmic organelles.[4,5]

Although autophagy is critical for the healthy long-term survival of mammalian cells, a partial reduction in autophagic turnover may constitute an oncogenic event. Insufficient autophagy stimulates oncogenesis and tumor progression through multiple non-exclusive mechanisms. Failure to activate the autophagic pathway may favor tumorigenic inflammation by stimulating necrotic cell death,[6] or by compromising the phagocyte-mediated removal of dying cells.[7] Moreover, deficient autophagy may promote genomic instability by compromising the fidelity of genome duplication, chromosome separation and/or DNA repair.[8,9]

A plethora of oncogenes (e.g., Akt1; phosphatidylinositol 3-kinase, PI3K; antiapoptotic proteins from the Bcl-2 family such as Bcl-2, Bcl-X_L and Mcl-1) suppress autophagy.[10] Conversely, several tumor suppressor proteins (e.g., proapoptotic BH3-only members of the Bcl-2 family;[11] death-associated protein kinase 1, DAPK1; phosphatase and tensin homolog, PTEN, which antagonizes PI3K; tuberous sclerosis 1 and 2, TSC1 and TSC2; as well as LKB1/STK11) stimulate autophagy, implying that their deficiency decreases autophagy.[10] Beclin 1, which is indispensable for autophagy, acts as a haploinsufficient tumor suppressor protein, and other proteins essential for autophagy (such as Atg4c, UVRAG and Bif-1) are also bona fide oncosuppressors.[12]

In contrast to the aforementioned tumor suppressors, recent findings indicate that p53 exerts a dual, apparently contradictory role in the control of autophagy. Whereas nuclear p53 can indeed act as an autophagy-promoting transcription factor,[13] it inhibits autophagy from a cytoplasmic localization.[14,15] In this context, the MDM2-dependent degradation of p53 represents a conditio sine qua non for

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the initiation of autophagy, at least in response to nutrient starvation, tunicamycin, rapamycin and lithium.[14,15] Accordingly, the complete depletion of p53 by gene knockout or RNA interference (RNAi) suffices to stimulate autophagy, both in normal and transformed cells of murine or human origin.[14,15] In human cancers, p53 is mostly inactivated by mutations that abolish its activity as a transcription factor, and often convert it into a dominant-negative variant that interferes with the functions of the wild type (WT) protein.[16-18] As a result of oncogenic stress and other poorly understood mechanisms, mutant p53 protein is often overexpressed by tumors, meaning that p53 mutations in clinical samples can be identified indirectly, through immunohistochemical detection of cancer cells that contain abnormally high levels of the protein.[16-18] Although such studies have generally visualized p53 mutants only in the nucleus of tumor cells, several in vitro large-scale analyses (based on immortalized cancer cell lines) have demonstrated that many p53 variants can indeed be found in either the nucleus and the cytoplasm.[19] Here, we addressed the question whether mutant p53 might inhibit or stimulate autophagy.

Results and Discussion

p53 mutants inhibit autophagy as a function of their cytoplasmic localization. Approximately 30% of *p53*$^{-/-}$ human colon carcinoma HCT 116 cells exhibit a punctuate, cytoplasmic distribution of GFP-LC3, which is indicative of increased levels of baseline autophagy. In these cells, plasmid-driven expression of p53^{WT} decreases autophagy, whereas the transfection with an empty vector (i.e., pcDNA3.1) fails to display any inhibitory effect.[14,15] As assessed by immunofluorescence microscopy, p53^{WT} reintroduced into *p53*$^{-/-}$ HCT 116 cells displayed an exclusively nuclear localization in ~40% of the cells (in which the levels of autophagy remained unchanged), while it showed a preferentially cytoplasmic pattern in the rest of the population (~60% of the cells, in which baseline autophagy was inhibited) (Fig. 1A and B).

Among a panel of cancer-associated p53 variants (each characterized by a single amino acid substitution) that were transfected into *p53*$^{-/-}$ HCT 116 cells, some behaved like p53^{WT} in showing either a nuclear or a cytoplasmic distribution (e.g., p53^{R175H}), while others were near-to-always found in the nucleus (e.g., p53^{P151H}) or in the cytoplasm (e.g., p53^{R273H}). Such nucleo-cytoplasmic distribution pattern was determined by immunofluorescence microscopy assessments (Fig. 1A and B), and confirmed by immunoblot detection of p53 upon subcellular fractionation (Fig. 1C). Twenty-two point mutants of p53 were investigated with respect to their subcellular distribution and their capacity to diminish baseline autophagy in *p53*$^{-/-}$ HCT 116 cells (Fig. 2). Interestingly, there was a significant correlation between the presence of p53 mutants in the cytoplasm (regardless of their concomitant localization in the nucleus) and their ability to repress autophagy. This was first observed within the entire populations of transfected cells (Fig. 2A), and was further corroborated when the subpopulations of cells that specifically displayed nuclear versus cytoplasmic p53 were studied (Fig. 2B). p53 variants that near-to-always localized in the nucleus (e.g., p53^{P151H} and p53^{R282W}) completely failed to repress autophagy, while mutants constantly characterized by a cytoplasmic distribution (e.g., p53^{A161T}, p53^{S227R}, p53^{E258K}, p53^{R273H} and p53^{R273L}) were highly efficient autophagy inhibitors (Fig. 2). The cytoplasmic localization of p53 isoforms that were expressed partially in the nucleus and partially in the cytoplasm (e.g., p53^{P98S}, p53^{K120D}, p53^{V143A}, p53^{R175C}, p53^{R175D}, p53^{R175H}, p53^{R175P}, p53^{R181H}, p53^{L194F}, p53^{S227K}, p53^{G245C}, p53^{R248L}, p53^{R248W}, p53^{R249S} and p53^{R280K}) exhibited a good correlation with their capability to inhibit autophagy, irrespective of their concomitant presence in the nucleus (Fig. 2A). Moreover, individual cells that exclusively expressed any among these mutants in the nucleus displayed either a normal degree of autophagy, similar to untransfected or empty vector-transfected *p53*$^{-/-}$ HCT 116 cells, or enhanced autophagy (Fig. 2B). In contrast, single cells in which p53 was restricted to the cytosol tended to manifest inhibited autophagy, independently from the specific p53 mutation (Fig. 2B). In summary, there was a strong correlation between the presence of normal or mutated p53 in the cytoplasm and autophagy inhibition, which was independent from p53 nuclear localization.

Cytoplasmic localization is the preponderant requirement for p53-mediated autophagy inhibition. Based on the fact that several cancer-associated mutations of p53 disrupt its transcriptional activity,[16-18] we suspected that removal of essential regions of the protein required for DNA binding (e.g., deletions ΔII, ΔIII, ΔIV and ΔV)[28] would not affect the autophagy-inhibitory function of p53. Indeed, all deletions affecting DNA binding were indistinguishable from p53^{WT} with respect to their capacity to repress autophagy upon transfection into *p53*$^{-/-}$ HCT 116 cells (Fig. 3), as well as to their nucleo-cytoplasmic distribution (data not shown). In contrast, the deletion of the nuclear export signal (NES) from the C terminus of p53, a manipulation which leads to the confinement of p53$^{\Delta NES}$ in the nucleus,[15] totally abolished its ability to reduce autophagy (Fig. 3). This contrasts with the intact autophagy-inhibitory potential of a p53 mutant that lacks the nuclear localization sequence (p53$^{\Delta NLS}$) and that is hence confined to the cytoplasm[15] (Fig. 3), further underscoring that only extranuclear p53 can repress autophagy. Altogether, these results corroborate the hypothesis that the maintenance of a cytoplasmic pool of p53 is essential for inhibiting autophagy.

Materials and Methods

Cell lines and culture conditions. WT and *p53*$^{-/-}$ HCT 116 colon carcinoma cell lines[20] were routinely maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM HEPES buffer, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (37°C–5% CO_2). Media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, USA).

Plasmids and transfection. Transient transfections were performed with Lipofectamine^{TM} 2000 reagent (Invitrogen), as recommended by the manufacturer, and plasmid-driven protein expression was allowed to proceed for 24 h before further experimental procedures. Cells were co-transfected with a plasmid for the expression of a green fluorescence protein (GFP)-LC3 fusion protein[21,22] and the empty vector pcDNA3.1 (negative control, from Invitrogen) or a construct encoding: (i) p53^{WT}; (ii) p53 variants that are retained in the cytoplasm or blocked in the nucleus due to the absence of the nuclear localization sequence (p53$^{\Delta NLS}$) or of the nuclear export signal (p53$^{\Delta NES}$),[15] respectively (kindly provided by Dr. C.G. Maki); (iii) p53 mutants exhibiting the following single amino acid substitutions: P98S, K120D,[23] V143A,[24] P151H, A161T, R175C, R175D,

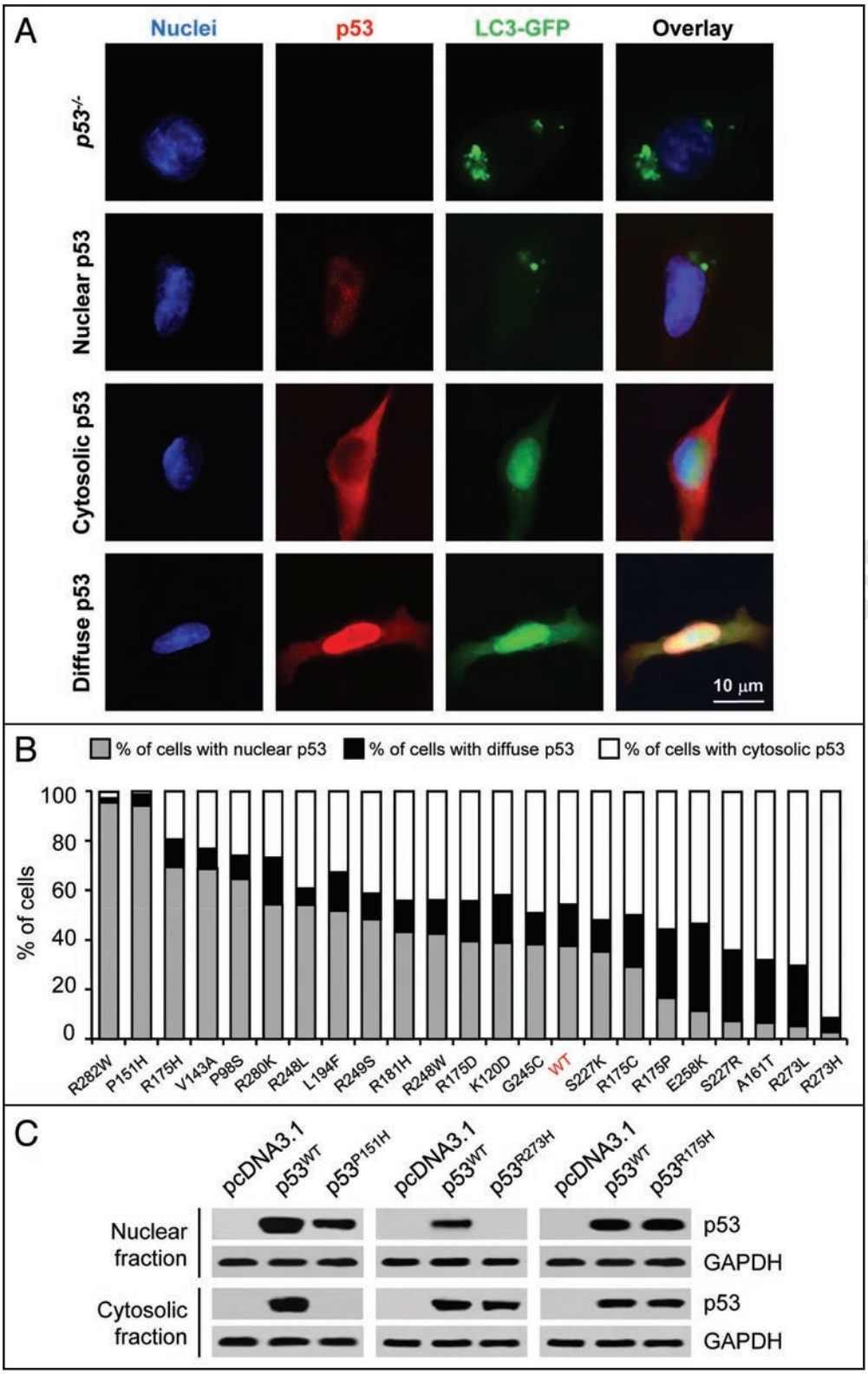

Figure 1. Subcellular distribution of p53 mutants. *p53*$^{-/-}$ HCT 116 cells were co-transfected with a plasmid for the expression of GFP-LC3 together with the empty vector pcDNA3.1 or a construct encoding wild type p53 (p53^{WT}) or p53 variants characterized by the indicated amino acid substitutions. Twenty-four hours later, the subcellular localization of p53 was determined by immunofluorescence microscopy (A and B) or subcellular fractionation followed by immunoblotting (C), whereas the aggregation state of GFP-LC3 was assessed by fluorescence microscopy (A). Upon reintroduction into *p53*$^{-/-}$ HCT 116 cells, some p53 mutants (e.g., p53^{P151H}) exhibited a nuclear localization in near-to-all cells, while others (e.g., p53^{R273H}) were almost invariably found in the cytoplasm. In addition, some p53 isoforms (e.g., p53^{R175H}) behaved as p53^{WT} and were found in either the nucleus or the cytoplasm, or distributed throughout the entire cell, although with different percentages (A–C). In non autophagic cells, GFP-LC3 emits a diffuse fluorescence from the entire cell (including the nucleus), whereas it manifests with bright cytoplasmic dots (which correspond to autophagic vacuoles) when autophagy takes place (A). (A) reports representative fluorescence patterns of p53 and GFP-LC3 in cells subjected to nuclear counterstaining with Hoechst 33342. The white bar illustrates picture scale (10 μm). In (B), grey, white and black columns illustrate the percentage of *p53*$^{-/-}$ HCT 116 cells (mean values, n = 3 independent experiments) exhibiting merely nuclear, purely cytosolic, or cytosolic plus nuclear (diffuse) p53, respectively, after transfection with the different p53 mutants included in this study. In (C), the results of subcellular fractionation followed by immunoblotting assessments are shown, which aimed at confirming the nucleo-cytoplasmic distribution of selected p53 mutants.

R175H,[25] R175P, R181H, L194F, S227K, S227R, G245C, R248L, R248W,[26] R249S,[27] E258K, R273H,[26,27] R273L, R280K, R282W (from Dr. T. Soussi's laboratory); (iv) p53 variants characterized by the following amino acid deletions: ΔII (117–142), ΔIII (171–181), ΔIV (234–258) and ΔV (270–286)[28] (from Dr. T. Soussi's laboratory).

Immunofluorescence microscopy. For the detection of p53 by immunofluorescence microscopy, cells were fixed and permeabilized as previously reported,[29] followed by incubation with specific primary antibodies. In particular, the antibodies Ab-2 or Ab-6 (Oncogene Science, Tarrytown, USA) were used to visualize p53^{WT} and p53 point mutants, whereas the DO-7 antibody (#SC-47698, Santa Cruz Biotechnology, Santa Cruz, USA) was employed to detect p53 deletion mutants. Goat anti-mouse IgGs coupled to Alexa Fluor® 568 fluorochrome (which emits in red; Molecular Probes, Invitrogen) were employed for revelation. Nuclei were counterstained in blue with 10 μg/ml Hoechst 33342 (Molecular Probes, Invitrogen). Cells exhibiting a diffuse distribution of GFP-LC3 (which fluoresces green) in both the cytoplasm and nucleus were considered as non-autophagic, while cells that displayed several intense GFP-LC3 aggregates in the

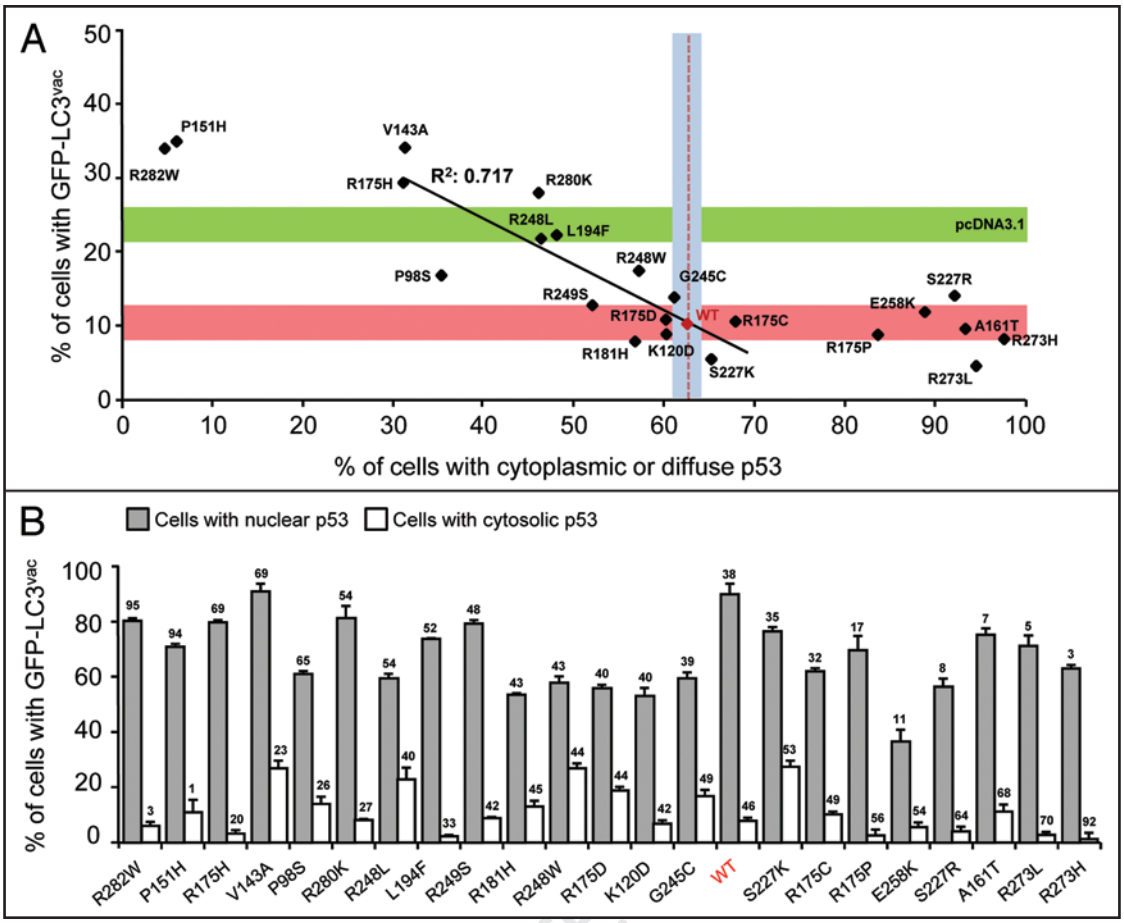

Figure 2. Effects of cancer-associated point mutations of p53 on its autophagy-inhibitory potential. *p53*$^{-/-}$ human colon carcinoma HCT 116 cells were co-transfected with a construct encoding GFP-LC3 plus the empty vector pcDNA3.1 or a plasmid for the expression of the indicated isoform of p53. Twenty-four hours later, the subcellular localization of p53 and GFP-LC3 aggregation were assessed by (immuno)fluorescence microscopy. All experiments were performed in (at least) n = 3 independent instances (A and B). For each p53 variant (here symbolized by a small rumble), panel A depicts the percentage of cells characterized by GFP-LC3 aggregates (GFP-LC3^{vac}) as a function of the percentage of cells that express p53 either in the cytoplasm or in both the cytoplasm and the nucleus (mean values). The horizontal green stripe depicts the percentage of *p53*$^{-/-}$ HCT 116 cells with GFP-LC3^{vac} (mean ± SEM) as observed after transfection with the empty vector pcDNA3.1 (negative control). The percentage of *p53*$^{-/-}$ HCT 116 cells displaying GFP-LC3^{vac} (mean ± SEM) recorded upon plasmid-driven expression of wild type p53 (p53^{WT}) is indicated by the horizontal red band. The vertical blue stripe recalls the percentage of p53^{WT}-transfected *p53*$^{-/-}$ HCT 116 cells (mean ± SEM) characterized by cytoplasmic p53^{WT} (see also Fig. 1B). The black line depicts the linear regression of data obtained for those p53 mutants that exhibited a cytoplasmic or diffuse localization in 20–80% of the cells. The corresponding coefficient of determination (R^2) is indicated. (B) illustrates the percentage of cells with GFP-LC3^{vac} (mean ± SEM), as quantified in the subpopulations of cells that exhibited merely nuclear (grey columns) versus strictly cytoplasmic (white columns) p53. The percentage of cells that expressed transfected p53 limitedly to the nucleus or to the cytoplasm is indicated above the corresponding columns (mean values, see also Fig. 1B). Please note that 100% is not systematically reached due to the fraction of cells in which p53 exhibited a diffuse, nuclear plus cytoplasmic localization. Please note also that pairwise comparisons between the levels of autophagy as recorded in the subpopulations of cells expressing nuclear versus cytoplasmic p53 always yielded *p* values <0.05 (Student's *t* test).

cytoplasm (GFP-LC3^{vac}) were classified as autophagic. Quantitative assessments and image acquisition were performed by means of an IRE2 microscope (Leica Microsystems, Weitzler, USA) equipped with a DC300F camera or with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Subcellular fractionation and immunoblotting. Upon trypsinization, cells were washed once in cold phosphate-buffered saline (PBS), lysed and subjected to subcellular fractionation by means of the CelLytic^{TM} NuCLEAR^{TM} Extraction kit (Sigma-Aldrich), following the manufacturer's recommendations. 50 µg of nuclear or cytosolic protein extracts were separated according to molecular weight on 4–12% SDS-PAGE precast gels (Invitrogen), followed by electrotransfer to Immobilon^{TM} polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). Unspecific binding sites were saturated by incubating membranes for 1 h in TRIS-buffered saline (TBS) supplemented with 0.05% Tween® 20 (Sigma-Aldrich) and 5% non-fat dry milk, and then membranes were incubated overnight at +4°C with a mouse monoclonal antibody specific for p53 (DO-1, #SC-126; Santa Cruz Biotechnology). Finally, revelation was performed by means of anti-mouse horseradish peroxidase-conjugated rabbit IgG (Southern Biotech, Birmingham, USA) and the SuperSignal West Pico chemoluminescent substrate (Pierce Biotechnology, Rockford, USA). A mouse monoclonal antibody that recognizes glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #MAb374) (Chemicon International, Temecula, USA) was employed to ensure equal loading of lanes.

Statistical procedures. Statistical analyses were carried out with Prism 3.03 (GraphPad Software Inc., San Diego, USA) and

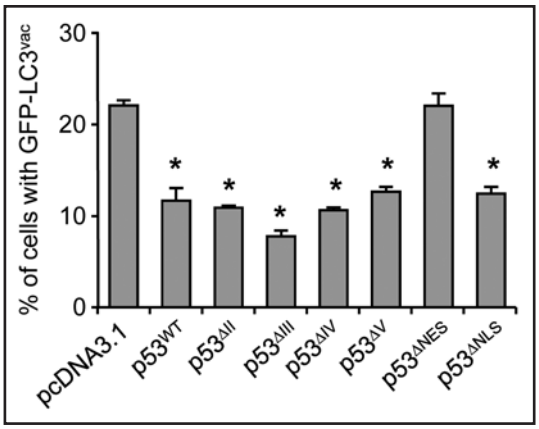

Figure 3. Effects of amino acid deletions on p53-mediated autophagy suppression. *p53*$^{-/-}$ human colon carcinoma HCT 116 cells were co-transfected with a GFP-LC3-encoding plasmid plus the empty vector pcDNA3.1 (negative control) or a construct for the expression of wild type p53 (p53^{WT}) or the indicated p53 deletion mutants. These mutations either affected the DNA-binding domain of p53 (e.g., ΔII, ΔIII, ΔIV, ΔV) or resulted in the inactivation of its nuclear export signal (i.e., ΔNES) or of its nuclear localization sequence (i.e., ΔNLS). After 24 h, the aggregation state of GFP-LC3 was quantified by immunofluorescence microscopy. Columns report the percentage of cells characterized by GFP-LC3 aggregates (GFP-LC3^{vac}, mean ± SEM, n = 3 independent experiments). Asterisks indicate statistically significant differences ($p < 0.05$) in baseline autophagy, as compared to empty vector-transfected *p53*$^{-/-}$ HCT 116 cells.

Microsoft Office 2003 Excel (Microsoft Corporation, Redmond, USA) software packages. In all experimental conditions, the percentage of cells exhibiting GFP-LC3^{vac} cells was quantified by two investigators (E.M. and E.T.) independently from each other, and is here reported as mean value ± SEM (n = 3 independent experiments). Statistical differences were evaluated by using unpaired Student's *t* tests ($p < 0.05$).

Concluding Remarks

The results contained in this article comfort the general hypothesis that cytoplasmic (but not nuclear) p53 exerts a tonic inhibitory effect on autophagy and that its subcellular localization of p53 is (one of) the major determinant(s) of this activity. Several p53 mutants that are associated with oncogenic transformation and that have been detected in human cancers can inhibit autophagy, in spite of the fact that most of them have lost their transactivating activity, or even act as dominant-negative inhibitors of p53^{WT} with respect to the induction of cell cycle-arresting and/or apoptosis-inducing transcriptional programs.[16-18] The capacity of different p53 variants to repress autophagy strictly correlated with their presence in the cytoplasm. When expressed in *p53*$^{-/-}$ HCT 116 cells, p53 mutants that exclusively localized to the nucleus failed to inhibit autophagy, whereas those variants that exhibited a mostly cytoplasmic localization were particularly efficient in suppressing the autophagic pathway. One limitation of this study is that p53 mutants were reintroduced only into a single cancer cell line (*p53*$^{-/-}$ colon carcinoma HCT 116 cells). Since it is known that the genetic background (including aberrations affecting DNA damage checkpoint proteins and cell cycle regulators like p16^{INK4a} and MDM2) may influence the stability and subcellular localization of p53,[19,30,31] we are far from assuming that the pattern of subcellular localization we observed for distinct p53 variants is a general one. Nevertheless, this makes the point for the purpose of the present report: several hot-spot mutants of p53 (e.g., those affecting R175, G245, R248, R249 and R273) retain their ability to suppress autophagy in spite of the fact that the corresponding amino acid substitutions have led to a loss-of-function phenotype with respect to the tumor suppressive function of p53.

Many among the point mutations evaluated in this study affect the DNA binding properties of p53,[16-18] which is obviously the case also for deletions of wide stretches of the p53 DNA-binding site (e.g., amino acids 117–142, 171–181, 234–258 and 270–286, which are missing from p53$^{\Delta II}$, p53$^{\Delta III}$, p53$^{\Delta IV}$ and p53$^{\Delta V}$ variants, respectively).[28] Nevertheless, such mutations or deletions did not affect the autophagy-inhibitory function of p53, underscoring that additional, yet-to-be-discovered molecular features of p53 account for autophagy suppression. Of note, several residues contained in the DNA-binding domain of p53 have also been found to be required for the interaction with Bcl-2 family proteins.[32,33] In particular, it has been shown that the deletion of amino acids 239–248 as well as the point mutations R175H, L194F and R273H abolish the capacity of p53 to co-immunoprecipitate with Bcl-2 or Bcl-X_L and to induce apoptosis by direct mitochondrial membrane permeabilization.[32-35] However, as shown here, p53^{R273H} maintains an autophagy-inhibitory potential, indicating that the two cytoplasmic actions of p53, namely induction of mitochondrial apoptosis and suppression of the autophagic program, involve different domains of the protein.

Enhanced autophagy in p53-deficient cells ameliorates their resistance to hypoxia and culture in nutrient-free conditions (as compared to cells that express p53^{WT}), presumably because this facilitates the preservation of high ATP levels due to the breakdown of energy-rich cellular macromolecules.[15,36,37] Hence, it is possible that the complete loss of p53 augments the fitness of cancer cells to adverse metabolic conditions (such as those found in poorly vascularized tumor cores). Nevertheless, as shown here, some cancer-associated p53 mutants that accumulate in the cytoplasm remain capable of suppressing autophagy. As mentioned in the Introduction, several oncoproteins do indeed inhibit autophagy while an expanding number of oncosuppressors stimulate the autophagic pathway. Taken together, these observations suggest a general scenario in which reduced (rather than enhanced) autophagy constitutes a hallmark of oncogenesis and tumor progression. Here, we show that some (but probably not all) cancer-associated p53 variants inhibit autophagy and hence—paradoxically—behave as oncogenes in this respect. It is tempting to speculate, yet remains to be proven, that this kind of p53 mutants may stimulate carcinogenesis in a double (or even triple) fashion, by combining defective transactivation of p53 transcriptional programs with suppressed autophagy (and in some instances also by interfering with the direct proapoptotic function of p53 at mitochondria).[32,35]

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