[Autophagy 4:6, 1-5; 16 August 2008]; ©2008 Landes Bioscience

Article Addendum

A dual role of p53 in the control of autophagy

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Abbreviations: GFP, green fluorescent protein; NF, nutrient-free medium; PI, propidium iodine; siRNA, small interfering RNA; WT, wild type

Key words: apoptosis, HDM2, DNA damage, ER stress, nutrient stress

Genotoxic stress can induce autophagy in a p53-dependent fashion and p53 can transactivate autophagy-inducing genes. We have observed recently that inactivation of p53 by deletion, depletion or inhibition can trigger autophagy. Thus, human and mouse cells subjected to knockout, knockdown or pharmacological inhibition of p53 manifest signs of autophagy such as depletion of p62/SQSTM1, LC3 lipidation, redistribution of GFP-LC3 in cytoplasmic puncta, and accumulation of autophagosomes and autolysosomes, both in vitro and in vivo. Inhibition of p53 causes autophagy in enucleated cells, indicating that the cytoplasmic, non-nuclear pool of p53 can regulate autophagy. Accordingly, retransfection of *p53*$^{-/-}$ cells with wild-type p53 as well as a p53 mutant that is excluded from the nucleus (due to the deletion of the nuclear localization sequence) can inhibit autophagy, whereas retransfection with a nucleus-restricted p53 mutant (in which the nuclear localization sequence has been deleted) does not inhibit autophagy. Several distinct autophagy inducers (e.g., starvation, rapamycin, lithium, tunicamycin and thapsigargin) stimulate the rapid degradation of p53. In these conditions, inhibition of the p53-specific E3 ubiquitin ligase HDM2 can avoid p53 depletion and simultaneously prevent the activation of autophagy. Moreover, a p53 mutant that lacks the HDM2 ubiquitinylation site and hence is more stable than wild-type p53 is particularly efficient in suppressing autophagy. In conclusion, p53 plays a dual role in the control of autophagy. On the one hand, nuclear p53 can induce autophagy through transcriptional effects. On the other hand, cytoplasmic p53 may act as a master repressor of autophagy.

Introduction

Cancer researchers have commonly considered that apoptosis (self-killing) and autophagy (self-eating) are mechanistically linked.[1,2] This global idea has apparently been corroborated by the observation that anti-apoptotic multidomain proteins of the Bcl-2 family inhibit autophagy[3] and that pro-apoptotic BH3-only proteins of the same family induce autophagy.[4,5] Moreover, several reports demonstrate that one of the principal pro-apoptotic transcription factors, p53, can stimulate autophagy[6-9] and that one of the p53-induced autophagy-inducing proteins, DRAM, also is required for p53-mediated cell killing.[8] Hence, many investigators have come to the conclusion that a range of overlapping stimuli, including chemotherapeutic agents that activate p53, can induce either of two catabolic processes, apoptosis or autophagy, both of which will contribute to the disappearance of tumor cells.

Although this undoubtedly applies to a variety of experimental setups, there is also evidence that autophagy can constitute a mechanism of cellular self-defense and that autophagy might actually prevent cell death in a variety of different situations, including nutrient starvation[10] or scarcity of essential growth factors,[11] hypoxia[12] and activation of p53.[7] Hence, attempts have been launched to use autophagy inhibitors for the treatment of cancer in mice.[7] Thus far, this approach is based on the use of rather non-specific agents (such as the lysomotropic agent chloroquine),[7] which may have autophagy-unrelated functions.[13] However, this illustrates a conceptual turning point beyond which autophagy has been considered predominantly as a cytoprotective (rather than as a cytocidal) mechanism.[14,15]

The tumor suppressor protein p53 is often inactivated in tumor cells, for instance due to mutations in p53 itself, due to mutations in the kinases that lead to its activation (such as ATM or Chk1) or due to the amplification of HMD2, the E3 ubiquitin ligase that ubiquitinylates p53 and targets it for proteasome-mediated degradation.[16,17] Hence, inactivation of the p53 system is one of the most frequent alterations that characterize cancer cells. Since p53 is involved in the induction of apoptosis and cell cycle arrest (which, when permanent is referred to as "senescence"), inactivation of the p53 system is linked to the relative resistance of tumor cells to respond to p53-activating agents, including a vast array of genotoxic agents that are used in chemotherapy.[16,17]

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Submitted: 05/26/08; Revised: 06/23/08; Accepted: 06/24/08

Previously published online as an *Autophagy* E-publication:
http://www.landesbioscience.com/journals/autophagy/article/6486

Recently, we discovered that inactivation of p53 induces autophagy.[18] This applies to a variety of methods for p53 inactivation: chemical inhibition with cyclic pifithrin-α (PFT-α), knockdown with small interfering RNAs specific for human p53, mouse p53 or the *Caenorhabditis elegans* p53 ortholog *cep-1*, or homologous recombination of p53 in human cancer cells, mice or nematodes. p53 inactivation was found to induce autophagy in multiple mammalian cell lines, namely in mouse embryonic fibroblasts (MEF), human HFFF2 fibroblasts, SH-SY5Y neuroblastoma, HeLa cervical cancer cells, HCT116[18] and RKO colon cancer cells (Fig. 1A and B), in vivo in multiple mouse tissues (pancreas, liver, kidney, heart) (Fig. 1C–E), as well as in *Caenorhabditis elegans* embryos[18] and adult pharyngeal cells (Fig. 1F and G). The signs of enhanced autophagy induced by p53 inactivation include transmission electron microscopy-detectable accumulation of autophagosomes and autophagolysosomes,[18] the aggregation of GFP-LC3 in cytoplasmic dots, both in cell lines (Fig. 1B) and in GFP-LC3-transgenic mice (Fig. 1D and E), maturation of LC3 from LC3-I to LC3-II (Fig. 1C), and the depletion of p62/SQSTM1, a protein whose abundance correlates inversely with autophagic activity.[19] These signs of increased autophagy persist in cell lines, in the presence of bafilomycin A_1 or cathepsin inhibitors,[18] suggesting that they truly involve an increased autophagic turnover. Ultrastructural data and immunofluorescence experiments indicate that p53 inactivation induces both autophagy of the endoplasmic reticulum (reticulophagy) and mitochondria (mitophagy). When p53 is inhibited in an acute fashion by addition of PFT-α, reticulophagy is induced more rapidly than mitophagy,[18] suggesting a relationship between p53 inhibition and ER stress (which also induces preferential reticulophagy).[20-22] Accordingly, p53 induces the phosphorylation of eIF2α, which is a hallmark of ER stress, and the knockdown or knockout of IRE1α, one of the quintessential ER stress effectors, abolishes autophagy induction by p53 inactivation.[18] Knockdown of essential autophagy genes such as *atg5, atg10, atg12, beclin 1* and *hvps34* also prevent autophagy induction by p53 inactivation.[18]

Altogether, these data indicate that p53 inactivation induces a potent autophagic response. Although this effect is found in multiple experimental systems, it is not a general phenomenon. For instance, in skeleton muscle cells from mice, the p53 knockout does not enhance autophagy.[18] Thus, there is some tissue specificity in the effect of p53 on autophagy that remains to be explained. However, in most tissues, p53 inactivation does induce autophagy. It is noteworthy that this effect is near-to-maximal in the sense that classical autophagy inducers such as culturing of cells in nutrient-free conditions, addition of rapamycin or lithium, or ER stress (induced by tunicamycin or thapsigargin) induce a similar level of GFP-LC3 puncta per cell (and a similar percentage of cells with GFP-LC3 puncta) as does the deletion, depletion or inhibition of p53.[18] Epistatic analysis in which p53 inactivation is combined with "classical" autophagy inducers reveals that there are no additive effects between the deletion, depletion or inhibition of p53, on the one hand, and nutrient-free culture, rapamycin, lithium or tunicamycin, on the other hand. GFP-LC3-transgenic mice kept in the absence of food for 24 hours manifests an increase in GFP-LC3 puncta in hepatocytes, pancreas epithelial cells and glomerular kidney cells.[18] This starvation-induced autophagy is only observed in p53-expressing ($p53^{+/+}$ or $p53^{+/-}$) mice. In p53-deficient ($p53^{-/-}$) mice, GFP-LC3 puncta are more frequent, and this increased autophagy was not further enhanced by starvation (Fig. 1C). Similar data are obtained in *C. elegans*. The knockout or knockdown of the p53 ortholog *cep-1* results in near-maximal autophagy (measured with the reporter gene dsRed::LGG-1) that is barely increased by starvation of the nematodes (Fig. 1G).[18]

p53 is best known as a transcription factor that operates in the nucleus, but it also acts in the cytoplasm, as an inducer of mitochondrial membrane permeabilization and apoptosis.[23-26] Indeed, p53 shuttles between the cytoplasm and the nucleus, thanks to the existence of nuclear import and export signals in the C terminus of the protein. Based on this dichotomy, we determined whether p53 inhibition by PFT-α would be able to induce autophagy in cytoplasts, that is, cells from which the nucleus has been removed. We found that PFT-α induces GFP-LC3 puncta with a similar efficacy in cells (with a nucleus) as in cytoplasts (without a nucleus), meaning that the nucleus is not required for the induction of autophagy by p53 inhibition.[18] Accordingly, p53 mutants that are purely nuclear (due to the deletion of a nuclear export sequence) are unable to inhibit autophagy when they were transfected into $p53^{-/-}$ cells, whereas p53 mutants that are purely cytoplasmic (due to the deletion of the nuclear import sequence) are fully competent in suppressing autophagy. Wild type p53 as well as cytoplasmic p53 mutants (but not nuclear p53 mutants) are able to reverse the hypophosphorylation of the mTOR substrate $p70^{S6k}$ and to abolish the hyperphosphorylation of AMP kinase (and that of the AMP kinase substrates TSC2 and AAC3) that normally characterize $p53^{-/-}$ cells.[18] These results indicate that cytoplasmic p53 inhibits autophagy, presumably through a (direct or indirect?) action on the mTOR/AMP kinase pathway.

The data mentioned above indicate that p53 depletion, deletion or inhibition cause autophagy, and that the mechanism elicited by p53 inactivation may operate within the "classical" autophagy pathway. We therefore asked whether "classical" inducers of autophagy would induce the degradation of p53, which in turn might facilitate autophagy. In accordance with this possibility, we found that numerous autophagy inducers including nutrient-free media, rapamycin, lithium or tunicamycin cause the rapid reduction of p53 levels, which can be inhibited by addition of chemical inhibitors of HDM2 (Nutlin-3 or RITA) or the 26S proteasome (MG132). Avoidance of p53 degradation simultaneously inhibited the induction of autophagy. Thus, inhibitors of HDM2 or the proteasome abolish the autophagic response to nutrient-free media, rapamycin, lithium or tunicamycin. These results have been obtained first in HCT116 and HeLa cells[18] and have subsequently been confirmed for RKO cells (Fig. 2A and B). Moreover, a p53 mutant that lacks the ubiquitination site for HDM2[27] is particularly efficient in preventing the induction of autophagy by nutrient-free media, rapamycin, lithium or tunicamycin.[18] Altogether, these data are compatible with the hypothesis that, at least in cell lines, autophagy induction requires the degradation of p53 as a necessary step for the de-inhibition of the autophagic machinery (Fig. 2C).

What might be the "advantage" for a tumor cell to inactivate the p53 system and hence to activate autophagy? There are (at least) two possibilities. First, since the loss of p53 is required for oncogenesis and tumor progression, enhanced autophagy due to p53 inactivation might be an undesired side effect (from the perspective of the tumor cell) that might be "corrected" by additional mutations or epigenetic changes including constitutive activation of the

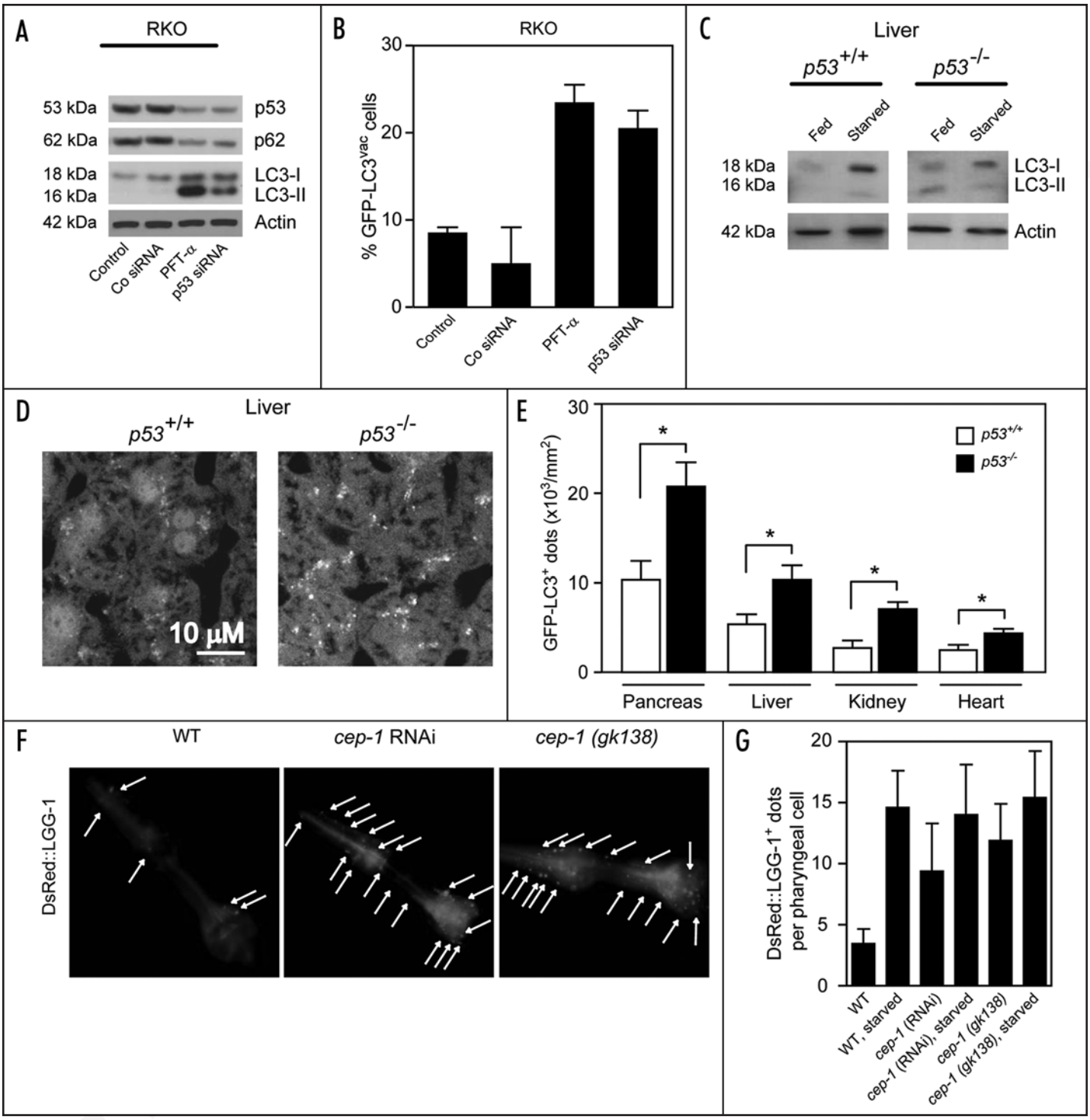

Figure 1. For figure legend, see page 3.

phosphatidylinositol-3 kinase/Akt pathway or loss of heterozygosity of *beclin 1* to inhibit excessive autophagy.[28] Second, an enhanced constitutive level of autophagy may actually be a way to improve the fitness of tumor cells. We found that $p53^{-/-}$ cells maintain their ATP levels when they are suddenly placed into a nutrient-free medium.[18] This differs from $p53^{+/+}$ cells that do manifest a sharp drop in their intracellular ATP concentration, which only recovers to normal levels after autophagy has been induced. This suggests that enhanced baseline autophagy, as manifest in $p53^{-/-}$ cells would allow the cells to maintain high levels of ATP in conditions in which the external supply of glucose and other substrates are interrupted. In accordance with this hypothesis, inhibition of autophagy by depletion of essential gene products (such as Atg5, Atg10 or Beclin 1), abolishes the capacity of $p53^{-/-}$ cells to maintain ATP levels in nutrient-free media.[18] Similar results are obtained when we measure cell death instead of ATP levels. The probability of survival is much higher for p53 knockout or knockdown cells as compared to their isogenic controls, when the cells are placed into a context of metabolic stress

Figure 1. Inhibition, depletion or deletion of p53 induces autophagy in human cells, mice or nematodes. (A and B) Induction of autophagy in RKO cells. Human RKO colon cancer cells were left untreated or cultured for 6 hours in the presence of a chemical p53 inhibitor, cyclic pifithrin α (PFT-α). Alternatively, the cells were transfected with a control siRNA (that targets emerin) or a p53-specific siRNA (as described in ref. 29), 48 hours before the abundance of p53, p62, LC3-I, LC3-II and actin was determined by immunobloting (A). Alternatively, cells were transfected 24 hours before the end of the experiment with GFP-LC3 and the percentage of cells exhibiting the accumulation of GFP-LC3 in puncta (GFP-LC3^{vac}) in which most of the GFP-LC3 accumulated in cytoplasmic dots was quantified by fluorescence microscopy (means ± SD of triplicates) (B). (C–E) Knockout of p53 induces autophagy in mice. Livers were obtained either from six-week old, female C57Bl/6 mice (either wild-type p53$^{+/+}$ or p53$^{-/-}$) that had been fed *ad libitum* or had been starved for 24 hours (from 10 p.m. to 10 a.m., with free access to water, however), followed by immunoblot detection of LC3-I and II (C). Alternatively, a ubiquitously expressed GFP-LC3 transgene was introduced into the p53$^{+/+}$ or p53$^{-/-}$ background and the number of GFP-LC3 puncta was quantified by fluorescence microscopy on tissue sections (D and E). Results in e are shown as means ± SD of three independent animals each. (F and G) Knockout or knockdown of the p53 orthologue *cep-1* induces autophagy in nematodes. Representative pharyngeal cells are shown for adult wild-type (WT) animals, nematodes that received *cep-1*-specific interfering RNA, or *cep-1*-deficient animals that all express a DsRed::LGG-1 transgene. Arrows indicate DsRed::LGG-1 puncta (F). The average number of DsRed::LGG-1 puncta per pharyngeal cell (arithmetic mean ± SD) are shown for animals that have been fed on rich medium or worms that were transiently kept in distilled water, for 24 hours (G). These values were obtained from 3 independent experiments, with each experiment including at least 50 individual animals. For each animal at least 4 pharyngeal cells were counted, giving a total of at least 200 cell observations for each of the above measurements ($p < 0.005$, unpaired t test).

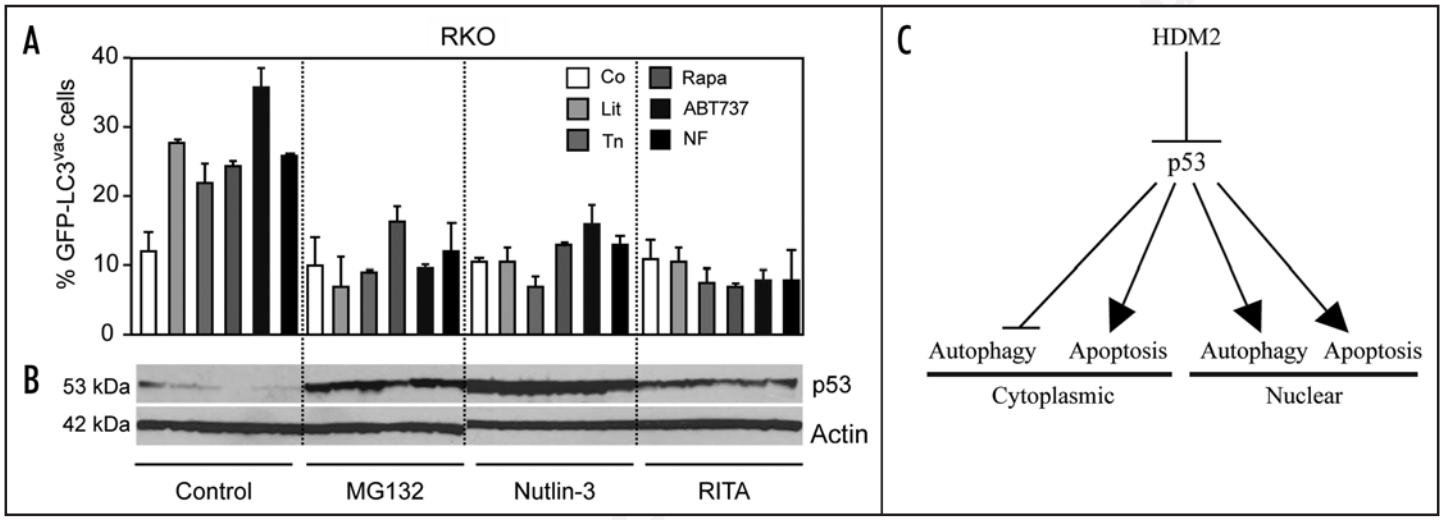

Figure 2. p53 degradation leads to the induction of autophagy. (A and B) Correlation of p53 expression and autophagy. RKO cells were transfected with GFP-LC3 and were treated 24 hours later for 6 hours with the indicated autophagy inducers (lithium, tunicamycin, rapamycin, ABT737 or nutrient-free culture conditions) either alone or in the presence of the proteasome inhibitor MG132 or the HDM2 inhibitors Nutlin-3 or RITA (for concentrations consult ref. 29). Then, the percentage (X ± SD, triplicates) of cells in which GFP-LC3 was concentrated in cytoplasmic puncta was determined among the GFP-expressing cells. The expression of p53 was also determined by immunoblot. Note that all autophagy-inducing regimens induce p53 depletion that can be avoided by MG132, Nutlin-3 or RITA. (C) Schematic representation of p53 effects on cells. As a transcription factor, p53 can activate genes that cause apoptosis or permanent cell cycle arrest. As a cytoplasmic protein, p53 can mediate a tonic inhibition of autophagy or the induction of apoptosis, the latter presumably through a direct effect on mitochondria.

(nutrient-free medium in the absence of oxygen), and this survival advantage disappears when autophagy is inhibited.[18]

Thus, p53 inactivation can ameliorate the capacity of cells to survive metabolic stress, due to an enhanced baseline autophagy. It is important to note that autophagy induction by p53 inactivation (measured as GFP-LC3 puncta) is a cell cycle-dependent phenomenon that occurs only during the G_1 and S phases of the cell cycle but not in the G_2 and M phases.[29] This applies to all autophagy inducers that we have tested in our laboratory, including culturing of cells in nutrient-free conditions or addition of rapamycin, lithium or tunicamycin.[29] Indeed, it would be counterintuitive that p53-deficient tumor cells would remain capable of proliferating if autophagy occurs at a constantly elevated level.

In conclusion, p53 turns out to be a central player in the regulation of autophagy. This discovery generates a series of important questions that one should tackle in the future: What are the exact molecular mechanisms through which cytoplasmic p53 represses autophagy? How does the cell "decide" between autophagy induction by nuclear p53 and autophagy inhibition by cytoplasmic p53? Do all p53 mutations that lose their transactivation function also affect the autophagy-inhibitory function of p53, or are there mutations that allow the distinct functions of p53 to be dissociated? What are the post-transcriptional modifications of cytoplasmic p53 that determine its autophagy-inhibitory or apoptosis-inducing potential? In which order are p53-inactivating and autophagy-inhibitory mutations acquired during the natural history of human cancer? And last but not least, would it be possible to ameliorate current cancer treatments by specifically inhibiting autophagy? We anticipate that the answers to these questions will have a major impact on the comprehension, diagnosis and treatment of neoplasia.

Acknowledgements

E.T. is a recipient of a PhD fellowship from Institut National contre le Cancer (INCa). N.T. is funded by EMBO and the EU 6th Framework Programme. F.C. is funded by Fondazione Telethon,

AIRC, Compagnia di San Paolo and the Italian Ministry of Health. G.K. is supported by Ligue contre le Cancer, Agence Nationale pour la Recherche (ANR), Cancéropôle Ile-de-France, European Commission (Active p53, Apo-Sys, Chemores, TransDeath, Right, Death-Train), Fondation pour la Recherche Médicale, and Institut National du Cancer (INCa).

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