

Report

Stimulation of autophagy by the p53 target gene Sestrin2

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The oncosuppressor protein p53 regulates autophagy in a dual fashion. The pool of cytoplasmic p53 protein represses autophagy in a transcription-independent fashion, while the pool of nuclear p53 stimulates autophagy through the transactivation of specific genes. Here we report the discovery that Sestrin2, a novel p53 target gene, is involved in the induction of autophagy. Depletion of Sestrin2 by RNA interference reduced the level of autophagy in a panel of p53-sufficient human cancer cell lines responding to distinct autophagy inducers. In quantitative terms, Sestrin2 depletion was as efficient in preventing autophagy induction as was the depletion of Dram, another p53 target gene. Knockout of either Sestrin2 or Dram reduced autophagy elicited by nutrient depletion, rapamycin, lithium or thapsigargin. Moreover, autophagy induction by nutrient depletion or pharmacological stimuli led to an increase in Sestrin2 expression levels in p53-proficient cells. In strict contrast, the depletion of Sestrin2 or Dram failed to affect autophagy in p53-deficient cells and did not modulate the inhibition of baseline autophagy by a cytoplasmic p53 mutant that was reintroduced into p53-deficient cells. We conclude that Sestrin2 acts as a positive regulator of autophagy in p53-proficient cells.

Introduction

Macroautophagy (which we refer to as “autophagy”) is a catabolic process in which portions of the cytosol or cytoplasmic organelles are sequestered within two-membraned vesicles, autophagosomes, and then targeted for bulk degradation through the fusion with lysosomes.^{1,2} This process can be elicited by multiple types of cellular stress, in particular nutrient depletion to which cells adapt by activating macroautophagy and by catabolizing macromolecules, allowing them to generate substrates for glycolysis and oxidative phosphorylation.³ The molecular pathway that links nutrient depletion to autophagy involves activation of the energy

sensor AMP-responsive protein kinase (AMPK, which responds to low ATP levels), which in turn inhibits the cardinal autophagy-repressive kinase, mammalian target of rapamycin (mTOR). Hence, inhibition of mTOR by addition of rapamycin is one of the most potent mechanisms to stimulate autophagy.⁴ Autophagy can also be elicited by alternative mechanisms, namely depletion of inositol-3-phosphate by addition of lithium or endoplasmic reticulum (ER) stress.⁵⁻⁸ Autophagy is indispensable for the adaptation of cells to stress, for the removal of damaged organelles, and for the maintenance of genomic stability.^{3,9,10} The invalidation of the autophagic program can accelerate tumor progression^{9,10} and disabled autophagy may constitute a near-to-general hallmark of developing cancer.^{11,12}

The oncosuppressor protein p53 plays an important role in the regulation of autophagy, at two radically distinct levels. At the first level, p53 can stimulate autophagy when it is present in the nucleus and transactivates autophagy-stimulating genes. Thus, the activation of p53 by genomic stress¹³ or an inducible p53 transgene^{14,15} can stimulate autophagy through transcriptional mechanisms. One p53-elicited gene product, Dram (for “damage-regulated autophagy modulator”) has been shown to be essential for p53-induced autophagy and apoptosis,^{14,15} as well as for autophagy induced by the p53 homolog p73.¹⁶ At a second level, p53 potently inhibits autophagy when it is present in the cytoplasm, where it inhibits AMPK and activates mTOR.¹⁷⁻²⁰ Thus, deletion, depletion or pharmacological inhibition of p53 triggers autophagy in normal and cancer cells.

Recently, Michael Karin and co-workers reported that the products of two p53 target genes, Sestrin1 and Sestrin2, inhibit mTOR through an indirect mechanism that involves the stimulation of AMPK.²¹ In view of the role of mTOR in the control of autophagy, we decided to investigate whether Sestrins might control autophagy. Here, we report that Sestrin2 acts as a new positive regulator of autophagy in p53-proficient but not in p53-deficient cells.

Results and Discussion

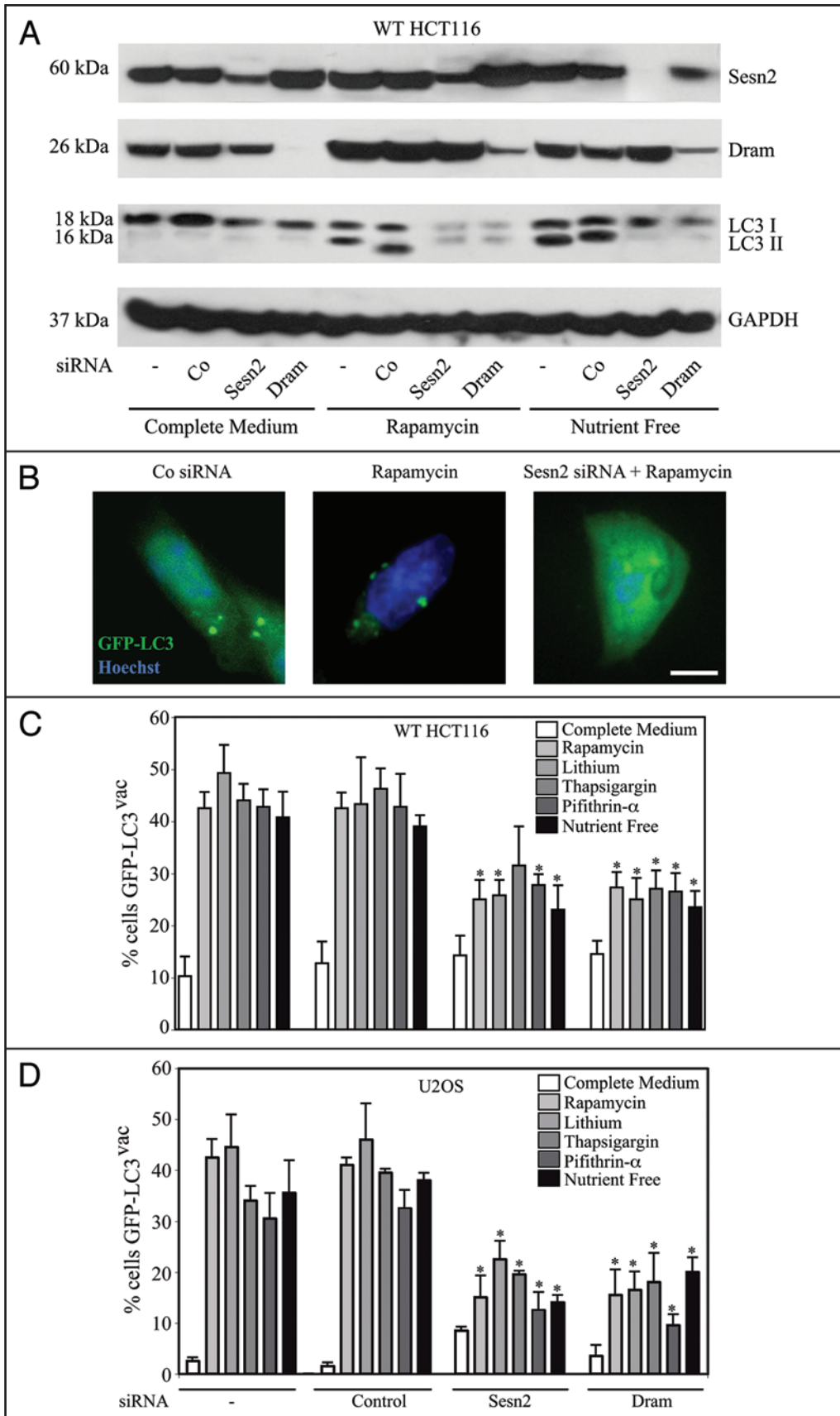
Contribution of sestrin2 and dram to the induction of autophagy in p53-proficient cells. To assess the possible contribution of Sestrins and Dram to the induction of autophagy, we

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Figure 1. Contribution of Dram and Sestrin2 to the induction of autophagy in p53-sufficient cells. (A) Immunoblot detection of LC3 conversion in HCT116 cells. Cells were subjected to the knockdown of Dram or Sestrin2 by siRNAs and 48 h later cells were stimulated overnight with rapamycin or culture in nutrient-free (NF) conditions for induction of autophagy. Representative immunoblots of Sestrin2 (Sesn2), Dram and LC3 are shown. GAPDH was detected as a loading control. (B and C) Fluorescence microscopic detection of GFP-LC3 aggregation in HCT116 cells. Cells were transfected with the indicated siRNAs, followed by transfection with GFP-LC3 at 24 h and overnight stimulation of autophagy starting at 48 h with the indicated agents. Representative fluorescence microphotographs are shown in (B) (the bar indicates 10 μ m) and quantitative results (means \pm SEM, n = 3) given in (C). Asterisks refer to significant (p < 0.01) inhibitory effects of Sestrin2 or Dram-specific siRNAs as compared to control siRNA. (D) Fluorescence microscopic detection of GFP-LC3 aggregation in U2OS cells. Identical setting as in (C) with the exception that U2OS cells were used.



took advantage of commercially available siRNAs that were used to efficiently deplete the respective proteins in a panel of cell lines comprising HCT116 colon cancer cells (Fig. 1A–C), U2OS osteosarcoma cells (Fig. 1D) or HeLa cervical carcinoma cells (not shown). This depletion did not affect the viability of cells, as determined by the combined assessment of plasma membrane permeability (with propidium iodine) and the mitochondrial transmembrane potential (with DiOC₆(3)) (data not shown).²² Once the depletion of Sestrin2 (Sesn2) or Dram was efficient, 48 hours after transfection, the cells were stimulated to activate the macroautophagic program by a series of different inducers, namely culture in nutrient-free (NF) conditions, addition of the quintessential mTOR inhibitor rapamycin, or addition of alternative pharmacological autophagy inducers: lithium,⁵ thapsigargin²³ or cyclic pifithrin- α (which is a chemical inhibitor of p53, yet may have additional off-target effects).^{24,25} Both nutrient depletion and pharmacological induction of autophagy resulted in an increased expression of Sestrin2 and Dram (Fig. 1A).

Autophagy was measured by two alternative methods, namely by immunoblot detection of the lipidation of LC3 (which leads to the conversion of the LC3 I isoform to the electrophoretically more mobile LC3 II isoform) or by assessing the redistribution of GFP-LC3 from a diffuse pattern to cytoplasmic puncta (which are indeed autophagosomes or autophagolysosomes the membranes of which are decorated by lipidated LC3).^{26,27} Using this technology, we found that depletion of Sestrin2 or knockdown of Dram were similarly efficient in preventing LC3 lipidation induced by nutrient deprivation or rapamycin (Fig. 1A). Similarly, we observed that the knockdown of Sestrin2 or Dram reduced the GFP-LC3 aggregation in cytoplasmic dots that was induced by the entire panel of chemical autophagy inducers (Fig. 1B–D). Similar results were obtained in three different p53-proficient cell lines, namely HCT116 cells (Fig. 1A–C), U2OS cells (Fig. 1D) and HeLa cells (not shown). In conclusion, both Sestrin2 and Dram contribute to the induction of autophagy in p53-proficient cells.

Absent contribution of sestrin2 and dram to autophagy induction in p53-deficient cells. We have shown in the past that distinct cell lines from murine or human origin activate the macroautophagic program when p53 is removed by homologous recombination or RNA interference.^{17–20} Accordingly, p53-deficient (p53^{-/-}) HCT116 cells exhibited a strong baseline level of autophagy, as indicated by the massive presence of LC3 II (Fig. 2A) and GFP-LC3-positive puncta (Fig. 2B). This level of autophagy could not be further enhanced by the addition of rapamycin or culture of cells in nutrient-free conditions (Fig. 2B). Transfection of p53^{-/-} HCT116 cells with wild type (WT) p53 caused an inhibition of autophagy. Similarly, a p53 mutant that lacks the nuclear localization sequence (NLS) and hence is confined to the cytoplasm was able to suppress the increased baseline autophagy of p53^{-/-} HCT116 cells. In contrast, a p53 mutant that lacks the nuclear export signal (NES) and hence is sequestered in the nucleus was incapable to inhibit autophagy, in accord with the notion that cytoplasmic (but not nuclear) p53 suppresses autophagy.^{17–20} In these experimental conditions p53^{-/-} HCT116 cells expressed significant levels of Sestrin2 and Dram without that WT, NLS⁻ or NES⁻ p53 would affect their abundance (Fig. 2A). Knockdown of

Sestrin2 or Dram failed to affect the levels of autophagy, as quantified by immunoblot (Fig. 2A) or fluorescence microscopy (Fig. 2B). Altogether, these data indicate that Sestrin2 and Dram do not participate in the regulation of autophagy by cytoplasmic p53.

Concluding Remarks

p53 regulates autophagy through two distinct mechanisms. On the one hand, the nuclear pool of p53 can induce autophagy through the transcriptional activation of specific target genes that include Dram (as previously shown by Kevin Ryan and collaborators)¹⁴ and Sestrin2 (as shown here). On the other hand, the cytoplasmic pool of p53 can inhibit autophagy by non-transcriptional mechanisms that are not fully understood. In this work, we depleted Dram or Sestrin2 from p53-proficient and p53-deficient cells to discover that both proteins contribute to the induction of autophagy, but only in p53-proficient cells. Thus, p53-proficient cells depleted from Dram or Sestrin2 exhibited a reduction in autophagy as compare to control cells, irrespective of whether autophagy was induced by the most physiological stimulus, nutrient depletion, or by pharmacological stimulators such as rapamycin. In strict contrast, we observed that Dram or Sestrin2 depletion failed to affect the increased autophagy of p53-deficient cells and had no effect on the inhibition of autophagy by cytoplasmic p53 that was re-introduced into p53-minus cells.

We conclude from these data that Dram and Sestrin2 are positive modulators of autophagy in p53-positive cells responding to distinct types of autophagy inducers. Indeed, when Dram or Sestrin2 were depleted, a whole series of distinct autophagy inducers elicited suboptimal responses. This applies to rapamycin but also to a variety of agents that cause autophagy through radically distinct mechanisms: lithium, thapsigargin and cyclic pifithrin- α . It should be noted that none of these inducers (nutrient depletion, rapamycin, lithium, thapsigargin or pifithrin- α) are known to activate p53, suggesting that other (transcriptional or post-transcriptional) p53-independent mechanisms likewise contribute to the modulation of Sestrin2 and Dram levels by autophagy inducers. At first glance, it may appear contradictory that the depletion of Dram or Sestrin2 reduces autophagy induction by the p53 inhibitor pifithrin- α , yet fails to inhibit autophagy induced by p53 deletion. This contradiction may be resolved by either assuming that pifithrin- α exerts off-target effects²⁶ or by speculating that the mechanisms of autophagy induction by acute pharmacological inhibition of p53 or deletion of the p53 gene from the genome may be distinct.

Irrespective of these theoretical considerations, our results indicate that Sestrin2 is a novel positive regulator of autophagy. In quantitative terms, Sestrin2 contributes to autophagy as much as does Dram, at least in the panel of human tumor cells investigated in this study. Hence, the elevation of Sestrin2 protein levels elicited via p53 or p53-independent mechanisms can efficiently stimulate autophagy.

Materials and Methods

Cell culture and autophagy induction. U2OS (osteosarcoma) and HeLa (cervical carcinoma) cells were cultured in DMEM containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate

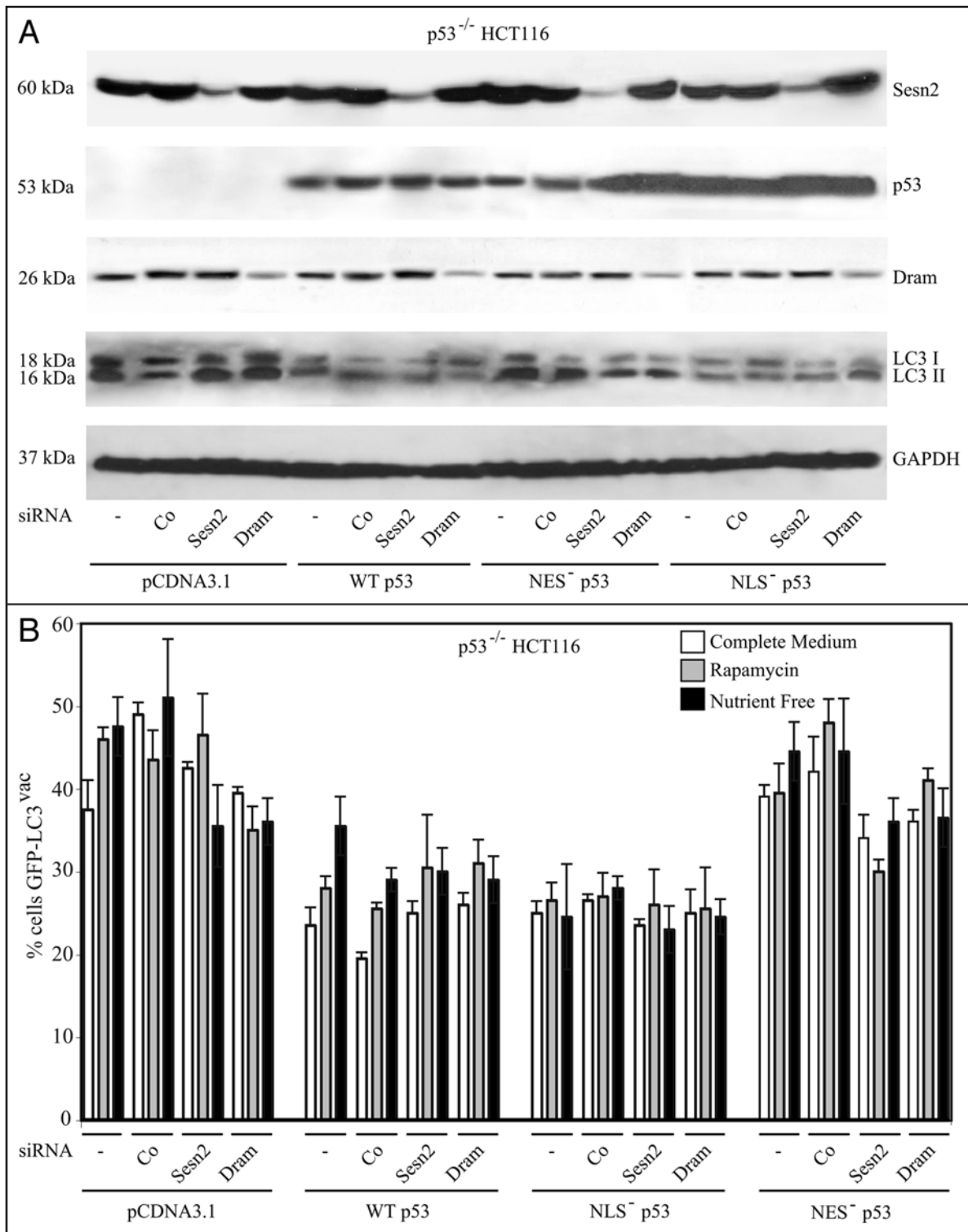


Figure 2. Failure of Dram and Sestrin2 to regulate autophagy in p53-negative cells. p53-deficient HCT116 cells were transfected with the indicated siRNAs, followed by transfection with different p53 constructs (wild type, NLS, NES), in the absence (A) or presence (B) of GFP-LC3 and immunoblot (A) or fluorescence microscopy (means \pm SEM, n = 3) (B) and addition of autophagy inducers overnight.

and 10 mM Hepes at 37°C under 5% CO₂. HCT116 colon carcinoma cell lines (wild type or p53^{-/-}, generous gift from Dr. B. Vogelstein)²⁸ were cultured in McCoy's medium containing 10%

FCS, 100 mg/l sodium pyruvate, 10 mM Hepes buffer, 100 units/ml penicillin G sodium and 100 μ g/ml streptomycin sulfate at 37°C under 5% CO₂. All media and supplements for cell culture

were purchased from Gibco-Invitrogen (Carlsbad, USA). For serum and amino acid starvation, cells were cultured in serum-free Earle's Balanced Salt Solution medium (Sigma-Aldrich, St. Louis, USA), which we refer to as nutrient-free medium (NF). Three x 10⁴ cells were seeded in 24 well plates and grown for 24 h before treatment with lithium chloride (Lit, 10 mM; Sigma-Aldrich), rapamycin (Rapa, 1 μ M; Tocris), pifithrin- α (PFT- α , 30 μ M; Calbiochem) or thapsigargin (Thapsi, 3 μ M; Calbiochem) for the indicated period (6 h, unless otherwise stated).

Plasmids, transfection and RNA interference. Cells at 80% confluence were transfected in 6-well plates with oligofectamine (Invitrogen) in the presence of 100 nM of siRNAs specific for human Sesn2 (Ambion, Applied Biosystems), Dram (Santa Cruz Biotechnology), a scrambled siRNA or a siRNA targeting the unrelated protein emerlin.²⁹ Transient transfections were performed with Lipofectamine 2000 (Invitrogen), and cells were used 24 h after transfection, unless indicated otherwise. Cells were transfected with an empty pcDNA3.1 vector alone or together with plasmids encoding GFP-LC3,³⁰ in the presence or absence of NES-p53, NLS-p53 (gift from Dr. C.G. Maki) or WT p53.

Quantification of autophagy. Autophagy was quantified by counting the percentage of cells showing accumulation of GFP-LC3 in vacuoles (GFP-LC3^{vac}, of a minimum of 100 cells per preparation in three independent experiments).^{26,27} Cells presenting a mostly diffuse distribution of GFP-LC3 in the cytoplasm and nucleus were considered non-autophagic, whereas cells representing several intense punctate GFP-LC3 aggregates with no nuclear GFP-LC3 were classified as autophagic. Each GFP-LC3 staining was read by two independent investigators. Cells were fixed with paraformaldehyde (4% w/v) for GFP-LC3 and immunofluorescence assays. Cells were stained for the detection of p53 (Oncogene Sciences) revealed by goat anti-mouse immunoglobulin Alexa[®] fluor conjugates (Molecular Probes). Nuclei were labeled with Hoechst 33342 (10 μ g/ml, Molecular Probes-Invitrogen). Fluorescence microscopy was analyzed with a Leica IRE2 microscope equipped with a DC300F camera.

Immunoblotting. Cells were lysed on ice in a buffer containing 1% NP40, 20 mM HEPES pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM dithiothreitol and 10 μ g/ml aprotinin, leupeptin, pepstatin before centrifugation (20 min, 14,000 rpm, 4°C) and collection of supernatant. Thereafter, protein extracts were separated on precast polyacrylamide gels (gradient 4%–12% or 10% gels, from Invitrogen), and subjected to standard immunoblotting protocols. To this aim, the following specific primary antibodies were employed: anti-p53 (DO-1; Santa Cruz), anti-LC3 (Cell Signaling), anti-Sestrin2 (Abcam) or anti-DRAM (Abcam) revealed with the appropriate horseradish peroxidase-labeled secondary antibodies (Southern Biotech, Birmingham, USA) and the SuperSignal West Pico chemoluminescent substrate (Pierce Biotechnology, Rockford). Anti-GADPH (AbCys, Paris, France) antibody was used to control equal loading.

Statistical analysis. Student's t-distribution probability density function was used for calculation of p values (*p < 0.01).

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