p53 induces a survival transcriptional response after nucleolar stress

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ABSTRACT Accumulating evidence indicates that increased ribosome biogenesis is a hallmark of cancer. It is well established that inhibition of any steps of ribosome biogenesis induces nucleolar stress characterized by p53 activation and subsequent cell cycle arrest and/or cell death. However, cells derived from solid tumors have demonstrated different degrees of sensitivity to ribosome biogenesis inhibition, where cytostatic effects rather than apoptosis are observed. The reason for this is not clear, and the p53-specific transcriptional program induced after nucleolar stress has not been previously investigated. Here we demonstrate that blocking rRNA synthesis by depletion of essential rRNA processing factors such as LAS1L, PELP1, and NOP2 or by inhibition of RNA Pol I with the specific small molecule inhibitor CX-5461, mainly induce cell cycle arrest accompanied by autophagy in solid tumor–derived cell lines. Using gene expression analysis, we find that p53 orchestrates a transcriptional program involved in promoting metabolic remodeling and autophagy to help cells survive under nucleolar stress. Importantly, our study demonstrates that blocking autophagy significantly sensitizes cancer cells to RNA Pol I inhibition by CX-5461, suggesting that interfering with autophagy should be considered a strategy to heighten the responsiveness of ribosome biogenesis–targeted therapies in p53-positive tumors.

INTRODUCTION

The synthesis of ribosomes is aberrantly increased in almost all cancers, and several studies have demonstrated that deregulation of ribosome biogenesis in cancer is not solely a consequence of increased proliferation, but is a key event in oncogenesis (Barna et al., 2008; Chan et al., 2011; Delloye-Bourgeois et al., 2012; Pelletier et al., 2018; Penzo et al., 2019). Biogenesis of ribosomes is initiated in the nucleolus by the transcription of a long 47S precursor ribosomal RNA (rRNA) that undergoes concomitant site-specific modifications, processing into mature 18S, 5.8S, and 28S rRNA species, and assembly with ribosomal proteins to form the 40S and 60S ribosomal subunits (Hadjiolova et al., 1993; Aubert et al., 2018; Bohnsack and Bohnsack, 2019). The fourth rRNA (5S), which is part of the 60S subunit, is transcribed independently by RNA Pol III (Ciganda and Williams, 2011). These steps involve a large assortment of processing and maturation factors in addition to the RNA and protein components of the ribosome itself. It is estimated that more than 200 additional accessory proteins and non-coding small RNAs engage in intricate reactions of processing, assembly and nuclear export to generate functional cytoplasmic 40S and 60S subunits (Bohnsack and Bohnsack, 2019). The integrity of the

nucleolus relies on the precise coordination of these reactions, and defects in any of these steps ultimately trigger a nucleolar stress characterized by a p53-regulated G1 cell cycle arrest and/or induction of apoptosis (Pestov *et al.*, 2001; Lohrum *et al.*, 2003; Rubbi and Milner, 2003; Zhang *et al.*, 2003; Bhat *et al.*, 2004; Dai and Lu, 2004; Dai *et al.*, 2004; Jin *et al.*, 2004; Yuan *et al.*, 2005; Chen *et al.*, 2007; Fumagalli *et al.*, 2009; Zhu *et al.*, 2009; Holzel *et al.*, 2010; Golomb *et al.*, 2014). Tumors appears to be more sensitive than normal cells to nucleolar stress, indicating that pathways involved in regulating ribosome production constitute a therapeutic vulnerability for cancers (Bywater *et al.*, 2012).

In general, activation of p53 represents a primordial response mechanism elicited by diverse type of stresses or loss of cellular homeostasis (Kastenhuber and Lowe, 2017; Zhang and Lozano, 2017). It is now well established that ribosomal proteins (RPs) play a critical role in mediating p53-signaling response triggered by nucleolar stress (Deisenroth *et al.*, 2016). One of the main mechanisms responsible for the activation of p53 after disruption of rRNA synthesis is the binding of unassembled RPs to MDM2, the E3 ubiquitin–protein ligase that normally targets p53 for proteasomal degradation in the absence of cellular stresses. Binding of free RPs to MDM2 inhibits its E3 ligase activity and consequently leads to the stabilization of p53. At least 16 different RPs from both the large and the small ribosomal subunits have been shown to bind to MDM2 (Chakraborty *et al.*, 2011). Among these, RPL11/uL5 and RPL5/uL18, as part of the 5S ribonucleoprotein (5S RNP), are absolutely essential to stabilize p53 after the induction of a nucleolar stress (Sun *et al.*, 2010; Bursa *et al.*, 2012; Fumagalli *et al.*, 2012; Sloan *et al.*, 2013).

p53 is well characterized by its ability to inhibit cell proliferation and induce apoptosis by transcriptional activation of the cyclin-dependent kinase inhibitor p21^{CIP1} and proapoptotic proteins, respectively. However, numerous studies have demonstrated that upon activation, p53 engages in an intricate anti-proliferative transcriptional program that spans a wide variety of biological processes including metabolism, reactive oxygen species (ROS) control, and autophagy (Nikulenkov *et al.*, 2012; Menendez *et al.*, 2013; Schlereth *et al.*, 2013; Allen *et al.*, 2014; Wang *et al.*, 2014). The p53-driven transcriptional program and its subsequent cellular outcome (survival or cell death) appear to be highly tissue-specific and dependent on the type of stress encountered. Studies examining the consequences of nucleolar stress after inhibition of rRNA synthesis in cells derived from solid tumors have demonstrated different degree of sensitivity where cytostatic effects rather than apoptosis are observed (Pestov *et al.*, 2001; Li *et al.*, 2016; Cornelison *et al.*, 2017; Ismael *et al.*, 2019). These effects have mainly been attributed to the transcriptional activation of p21^{CIP1}, which leads to a G1-cell cycle arrest with minimal cell death observed. Besides these observations, the extent to which the p53-specific transcriptional program is activated after nucleolar stress is currently unknown.

Given the growing interest in targeting ribosome synthesis as a novel therapeutic approach for cancer, it is becoming crucial to understand the p53 specific transcriptional response to nucleolar stress. Here, we utilized gene expression analysis to assess transcriptomic changes after inhibition of ribosome biogenesis. To identify p53-induced transcripts, the data were compared with published global run-on (GRO) sequencing/ChIP-seq studies of p53 targets (Nikulenkov *et al.*, 2012; Menendez *et al.*, 2013; Schlereth *et al.*, 2013; Allen *et al.*, 2014; Wang *et al.*, 2014) and verified in wild type (WT) vs. isogenic p53 –/– cells. We uncovered that nucleolar stress leads to the activation of a p53-transcriptional survival program that

includes genes involved in autophagy, metabolism, and ROS control. Importantly, inhibition of autophagy dramatically reduced the viability of cancer cells treated with the selective RNA pol I inhibitor CX-5461 (Drygin *et al.*, 2011; Bywater *et al.*, 2012). These results demonstrate that targeting p53-regulated pathways could enhance the efficacy of ribosome biogenesis drugs used for the treatment of cancers with active p53.

RESULTS

Inhibition of rRNA processing and ribosome biogenesis induces autophagy

Apart from the induction of the cyclin-CDK inhibitor p21^{CIP1}, very little is known about the cellular response and the p53-dependent transcriptional program initiated upon nucleolar stress. To further investigate how cells respond and adapt to nucleolar stress, we inhibited rRNA processing in HCT116 colon carcinoma cells (p53 WT) by RNAi depletion of LAS1L, an endoribonuclease essential for processing of the 28S rRNA and the synthesis of the 60S ribosomal sub-unit (Castle *et al.*, 2010, 2013; Schillewaert *et al.*, 2012; Gasse *et al.*, 2015). As expected, knockdowns of LAS1L lead to protein stabilization of p53, induction of p21^{CIP1}, and a G1-cell cycle arrest (Figure 1, A and B). Disruption of normal nucleolar morphology typical of nucleolar stress was also observed in LAS1L depleted cells (Figure 1C). In response to nucleolar stress, the ribosomal proteins RPL11/uL5 and RPL5/uL18 have been shown to be essential for p53 activation, as they bind to and inactivate MDM2, the E3 ubiquitin ligase that targets p53 for proteasome-mediated degradation (Sun *et al.*, 2010; Bursa *et al.*, 2012; Fumagalli *et al.*, 2012; Sloan *et al.*, 2013). Knockdown of both LAS1L and RPL11/uL5 abolished the stabilization p53 observed, indicating that inhibition of rRNA processing activates p53 through the sequestration of MDM2 by ribosomal proteins (Figure 1D).

Although depletion of LAS1L dramatically affects cell proliferation (Figure 2A), only a small but significant drop in cell viability is observed at day 5 after LAS1L knockdowns (Figure 2B), suggesting that p53 is not likely to activate the transcription of apoptotic genes in response to nucleolar stress. Instead, we observed a dramatic increase in cells undergoing autophagy as monitored by the relocalization of EGFP-LC3B into punctate autophagosomes by fluorescence microscopy (Figure 2, C and D). Taken together, these observations indicate that inhibition of rRNA processing triggers a classical nucleolar stress response whereby p53 protein levels accumulate as a result of MDM2 inhibition by unassembled ribosomal proteins. Activated p53 likely promotes a prosurvival stress response that involves the transcriptional programing of autophagy-promoting pathways.

p53 activates a survival transcriptional program in response to nucleolar stress

To investigate changes in gene expression induced by nucleolar stress, we performed gene expression analysis comparing RNA from HCT116 (p53 WT) cells treated with control (duplicate experiment) vs. LAS1L siRNAs (two different siRNAs). By setting an arbitrary fold change (FC), cutoffs of >2, and significance *p*-values of <0.01, we identified 157 up-regulated and 128 down-regulated genes in LAS1L-depleted cells (Figure 3A and Supplemental Table S1). Of these 157 up-regulated genes, 11 are known validated direct p53 targets and 51 are p53 targets predicted by one or more published microarray, ChIP-seq, or GRO-seq studies (Supplemental Table S2). A gene set enrichment analysis (GSEA) from the Molecular Signatures Database (Subramanian *et al.*, 2005; Liberzon *et al.*, 2011) revealed that up-regulated genes were enriched for p53 gene
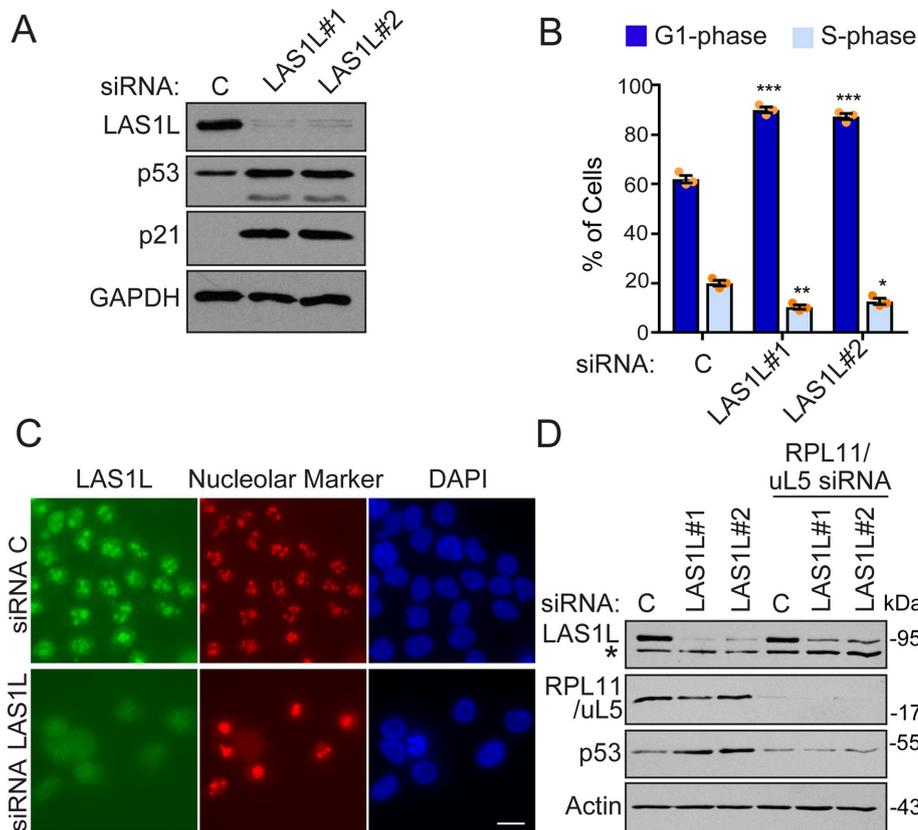
FIGURE 1: Inhibition of rRNA processing through depletion of LAS1L causes nucleolar stress. (A) HCT116 cells were transfected with control (represented by the letter C) or two different-LAS1L specific siRNAs. After 72 h, a portion of the cells were analyzed by Western blotting with the indicated antibodies. (B) RNAi-treated cells from C were labeled with BrdU and PI and analyzed by flow cytometry for DNA content. The results are graphed as percentages of cells in G1- and S-phase. The data are presented as means of three independent experiments ± SEM. Differences between groups (RNAi vs. nontargeting control) were evaluated using two-tailed Student t tests among replicate experiments. $**P = 0.003$, $*P = 0.012$: comparing to S-phase of the control; $***P < 0.001$: comparing to G1-phase of the control. (C) Immunofluorescence analysis of HCT116 cells transfected with control C or LAS1L #1 siRNA. Seventy-two h after transfection, cells were fixed and immunostained with anti-LAS1L (green) and anti-Fibrillarin (red nucleolar marker). DNA was visualized by staining with Hoechst 33342 (blue). Scale bar is representative of all panels: 10 μm. (D) HCT116 cells were transfected with C or two different LAS1L specific siRNAs with or without cotransfection of RPL11/uL5 siRNA. Seventy-two h later, cells were analyzed by Western blotting with the indicated antibodies. Nonspecific bands were annotated with *.

network, epithelium development, genotoxicity pathway, and metabolic processes including lipid binding and catabolic process/metabolism categories that comprise genes involved in promoting autophagy, such as *SESN2*, *MAPLC3B*, *WIPI1*, and *CCNG2* (Maiuri et al., 2009; Mourgues et al., 2015; Proikas-Cezanne et al., 2015; Bento et al., 2016; Figure 3, B and D). A closer inspection of the up-regulated gene list showed enrichment for cell cycle arrest, ROS control, cell migration, and noncoding RNAs (Figure 3D). Genes that were down-regulated showed enrichment for nitrogen compound biosynthesis, small molecule/organic acid metabolic processes, cell cycle, and nitrosative stress categories (Figure 3C). Genes enriched for cell cycle regulation included *CCNE2* (CYCLIN E2), *CDC25A*, and *E2F2* (Figure 3E), which is in accordance with the G1-cell cycle arrest observed upon LAS1L depletion (Figure 1B). Only a few apoptotic genes were up-regulated (*BAX*, *TP53I3*, *CFLAR*; Figure 3D) suggesting that instead of cell death, nucleolar

stress leads to p53-mediated metabolic pathway remodeling to support cell survival by promoting the maintenance of cellular energy levels.

To determine whether changes in metabolic gene expression observed after LAS1L depletion were associated with nucleolar stress in general, we tested for autophagy induction after RNAi knockdown of two other essential 60S ribosome biogenesis factors (PELP1 and NOP2; Hong et al., 1997; Finkbeiner et al., 2011; Castle et al., 2012; Bourgeois et al., 2015) and after inhibition of RNA Pol I with CX-5461. Similarly to depletion of LAS1L, knockdowns of PELP1 and NOP2, as well as inhibition of rRNA transcription by CX-5461, leads to p53 stabilization, induction of p21[CIP1], and down-regulation of cyclin E protein (Figure 4A). The well-characterized p53 target and positive regulator of autophagy SESTRIN 2 (Budanov et al., 2004; Budanov and Karin, 2008), whose gene expression (*SESN2*) was found increased in our gene expression analysis (Figure 3 and Supplementary Table S1), was also dramatically up-regulated at the protein level after inhibition of ribosome synthesis (Figure 4A). Autophagy is associated with conversion of the microtubule-associated protein 1 light chain 3 (LC3) from the cytosolic LC3-I to the autophagosome-associated LC3-II form (Bento et al., 2016). Increased levels of LC3-II were clearly detected in cells depleted of LAS1L, PELP1, or NOP2 and after inhibition of RNA Pol I by CX-5461 (Figure 4A). In addition, fluorescence microscopy showed the redistribution of EGFP-LC3 into punctate autophagosomes after nucleolar stress (Figure 4B and Supplemental Figure S1), suggesting that the increase in LC3B gene (*MAPLC3B*) expression observed is associated with induction of autophagy.

We next used qRT-PCR to confirm the gene expression results in selected subsets of genes from different biological processes categories found to be up-regulated by LAS1L depletion (Figure 3B). In agreement with the induction of autophagy observed in Figure 4, depletion of LAS1L, PELP1, and NOP2 or inhibition of rRNA transcription by CX-5461 leads to increased transcripts of *SESN2*, *MAP1BLC3*, and *CCNG2*, which are known to regulate autophagy positively (Maiuri et al., 2009; Mourgues et al., 2015; Bento et al., 2016; Figure 5A). As expected, p21[CIP1] (*CDKN1A*) transcript was up-regulated above twofold, whereas transcription of the proapoptotic gene *BAX* only showed a modest increase after nucleolar stress (Figure 5A). However, transcription of *TP53I3* (PIG3), another proapoptotic gene, was substantially increased, particularly in the CX-5461 treated cells (Figure 5A). Because PIG3 expression alone is insufficient to induce apoptosis (Polyak et al., 1997), this increase is unlikely to by itself override the overall transcriptional output of survival genes up-regulated by nucleolar stress. Genes involved in central metabolism regulation

A

B

C

siRNA C   siRNA LAS1L

EGFP-LC3B

D
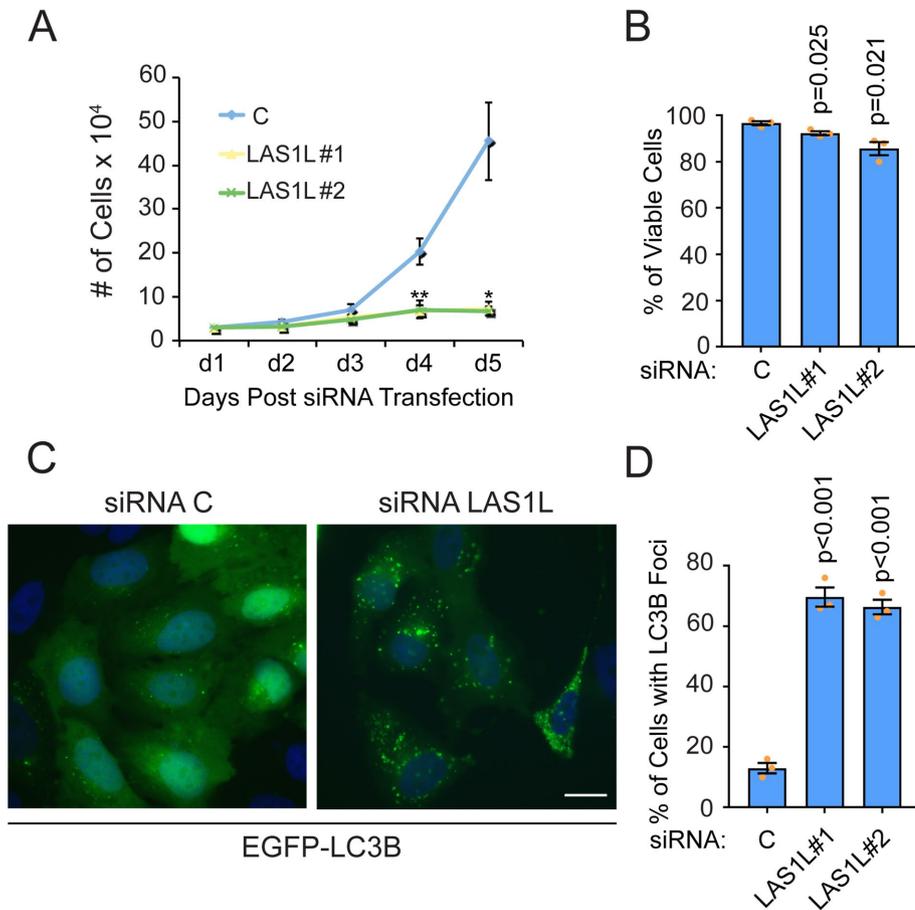
FIGURE 2: Inhibition of rRNA processing induces autophagy. (A) HCT116 cells were transfected with nontargeting control (C) or LAS1L#1 and LAS1L#2 siRNA. Twenty-four h after transfection, cells were replated and counted at days 2, 3, 4, and 5 posttransfection. Cell count is represented as number of cells/ml $\times 10^4$. The data are presented as means of three independent experiments ± SEM. *$P = 0.026$ (LAS1L#1 vs. C), $P = 0.024$ (LAS1L#2 vs. C), ** $P = 0.008$ (LAS1L#1 vs. C), $P = 0.002$ (LAS1L#2 vs. C). (B) Cells from A were harvested at day 5 and viability was determined by trypan blue exclusion. The data are presented as means of three independent experiments ± SEM. (C) HCT116 cells stably expressing EGFP-LC3B were transfected with C or LAS1L#1 and LAS1L#2 siRNA. Seventy-two h after, cells were fixed, stained with Hoechst 33342 (blue), and imaged by fluorescence microscopy (only the data from LAS1L siRNA #1 are represented). Scale bar is representative of all panels: 10 µm. The number of cells with more than 10 EGFP-LC3B foci was counted (200 cells per condition). (D) Quantification of the data from panel C. The data are presented as means of three independent experiments ± SEM. For all panels, statistical significance comparing knockdowns with C was calculated using two-tailed Student's *t* tests among biological replicates.

analysis comparing their expression level in isogenic HCT116 p53 +/+ and p53 –/– cell lines. Expectedly, inhibition of ribosome synthesis by LAS1L knockdown or treatment with CX-5461 induced the expression and stabilization of p21[CIP1] protein only in p53 WT cells (Supplemental Figure S2A). Additionally, the up-regulation of SESTRIN 2 and LC3B proteins was not observed in p53 null cells after ribosomal stress (Supplemental Figure S2B). Nucleolar stress increased the expression of *SESN2* (SESTRIN 2) as well as the expression of the predicted p53 target genes *CCNG2, ASS1, LOXL4, DGKA, BAX, FABP6, FGGY,* and *TP53I3* in a p53-dependent manner (Figure 6). Although *MAP1LC3B* (LC3B), *GSTT2, ALDH3B1, CKMT1A,* and *SLC1A3* have not been predicted by any microarray, ChIP-seq, or GRO-seq studies to be p53 targets, their up-regulation following inhibition of ribosome biogenesis was observed only in p53 WT cells, suggesting that their transcriptional regulation is p53-dependent (Figure 6). Whether they are direct transcription targets of p53 remains to be determined. Altogether, these results suggest that p53 activates the transcription of genes mainly involved in cell cycle arrest, metabolism, ROS control, and autophagy to promote the survival of cancer cells after nucleolar stress.

Inhibition of autophagy sensitizes p53 positive cancer cells to nucleolar stress

Increased ribosome biogenesis is a hallmark of cancer and inhibition of rRNA synthesis is now emerging as a prime therapeutic approach for cancer. The rDNA transcription inhibitor CX-5461 has been shown to have p53-dependent anti-tumorigenic activity in B-lymphoma models, and results of a phase I dose escalation study in patients with advanced hematologic cancer have shown antitumor activity in both WT and p53 mutant malignancies (Bywater *et al.*, 2012; Khot *et al.*, 2019). However, in some solid tumors, CX-5461 appears to have variable cytostatic effects characterized by the inhibition of cell growth and induction of autophagy (Drygin *et al.*, 2011; Li *et al.*, 2016; Cornelison *et al.*, 2017; Ismael *et al.*, 2019). In agreement with these observations, our data demonstrating that inhibition of ribosome synthesis induces the expression of autophagy promoting genes suggest that interfering with this pathway could reduce the survival of these cells after nucleolar stress. To test this, we used RNAi to knock down ATG7, an E1-like activating enzyme essential for autophagy, and SESTRIN 2, which was shown to promote autophagy by activating an AMPK-dependent inhibition of mTOR signaling (Tanida *et al.*, 1999; Komatsu *et al.*, 2001; Budanov and Karin, 2008; Maiuri *et al.*, 2009). As shown in Figure 7, A and B, depletion of SESTRIN 2 or ATG7 with two different siRNAs considerably increased the sensitivity of HCT116 cells to CX-5461 treatment, suggesting

(*ASS1, DGKA*; Merida *et al.*, 2008; Haines *et al.*, 2011), ROS control (*GSTT2*; Tew and Townsend, 2012), and, surprisingly, cell migration (*LOXL4, ANGPTL4*; Minn *et al.*, 2005; Payne *et al.*, 2005; Padua *et al.*, 2008) also showed higher transcript levels after inhibition of ribosome synthesis (Figure 5A). Interestingly, a subset of genes (*ALDH3B1, FABP6, CKMT1A, FGGY,* and *SLC1A3*) were only found to be up-regulated by depletion of LAS1L, PELP1, or NOP2 and not by inhibition of Pol I transcription by CX-5461 (Figure 5B). In addition to inhibition of Pol I activity, CX-5461 has also been shown to cause DNA damage by blocking replication forks and inducing single-strand DNA breaks, which could explain the differences in gene transcription observed (Xu *et al.*, 2017; Bruno *et al.*, 2020).

To assess whether the sets of genes found to be up-regulated after nucleolar stress are p53-regulated, we performed qRT-PCR

FIGURE 3: Nucleolar stress promotes the induction of a survival transcriptional program. (A–C) HCT116 cells were transfected with nontargeting control (in duplicate) or LAS1L#1 and LAS1L#2 siRNA. Seventy-two h later, total RNAs from two independent biological replicates were extracted and subjected to microarray analysis to identify transcriptional changes. The data were analyzed with an arbitrary fold change (FC) cut-offs of >2 and significance *p*-values of <0.01. A, scatterplot of all genes with significant change (*p* < 0.01). Genes found up-regulated are listed in B and C. Gene set enrichment analysis (GSEA) was determined using the Molecular Signatures Database for up-regulated and down-regulated genes separately using the ontology and canonical pathways gene sets. Overlaps were computed with an FDR *q*-value less than 0.05. The number of genes overlapping the particular term over the total term size is indicated in the bar plot. (D, E) Partial list of genes found up-regulated and down-regulated according to their cellular functions. Genes in bold represent known validated direct p53 targets and genes underlined are p53 targets predicted by one or more published microarray, ChIP-seq, or GRO-seq studies.

that autophagy is required for cell survival after inhibition of rRNA synthesis. To further confirm this, we next tested whether pharmacological inhibition of autophagy could also sensitize cells to nucleolar stress. Cells were treated concomitantly with CX-5461 and increasing doses of bafilomycin A1 (0, 50 nM, 100 nM), a macrolide antibiotic that reversibly inhibits late-phase autophagy, or chloroquine (0, 5 μM, 10 μM), which inhibits lysosomal hydrolases and prevents autophagosomal fusion and degradation of vesicle content. Although bafilomycin treatment by itself showed a moderate reduction in cell viability, cotreatment
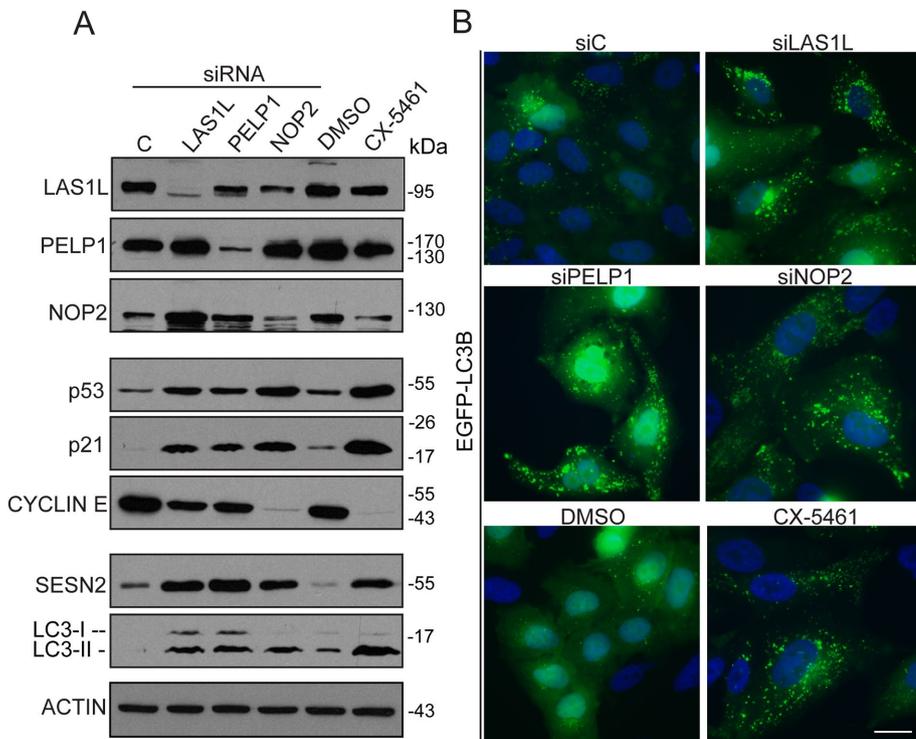
FIGURE 4: Nucleolar stress increases SESTRIN 2 protein level and promotes autophagy. (A) HCT116 cells were transfected with nontargeting control (siC), LAS1L siRNA #1, PELP1 siRNA, or NOP2 siRNA or treated with DMSO or 500 nM CX-5461. Seventy-two h later (for siRNA treatment) or 24 h later (CX-5461), cells were analyzed by Western blotting with the indicated antibodies. (B) HCT116 cells stably expressing EGFP-LC3B were transfected with nontargeting control (C) or LAS1L siRNA #1, PELP1 siRNA, or NOP2 siRNA or treated with DMSO or 500 nM CX-5461. Seventy-two h after (for siRNA treatment) or 24 h after (CX-5461), cells were fixed, stained with Hoechst 33342 (blue), and imaged by fluorescence microscopy to visualize the relocalization of EGFP-LC3B to punctate autophagosomes. Scale bar is representative of all panels: 10 μm.

DISCUSSION

The data presented in this study demonstrate that inhibition of ribosome biogenesis leads to the establishment of a survival transcriptional program characterized by cell cycle arrest and activation of autophagy-promoting pathways in p53-positive cancer cells. Our data also uncovered several genes involved in the regulation of central metabolism and ROS control that are up-regulated in a p53-dependent manner in response to rRNA synthesis inhibition. These findings suggest that inhibition of ribosome biogenesis elicits a transient metabolic stress whereby p53 regulates an adaptive transcriptional program by promoting catabolism, metabolic rewiring, and protection against ROS, while inhibiting cellular proliferation.

Although the roles of p53 have mostly been studied in response to genotoxic stress, it is now well established that p53 also plays a major function in the transcriptional regulation of metabolic homeostasis in response to energetic stress (Napoli and Flores, 2017; Labuschagne et al., 2018; Lacroix et al., 2020). For example, p53 has been shown to activate fatty acid oxidation (FAO) (Goldstein and Rotter, 2012) and rearrange arginine metabolism through direct transcriptional activation of carnitine palmitoyltransferases (CPT1A, CPT1C) and argininosuccinate synthase 1 (ASS1), respectively, which we found up-regulated in a p53-dependent manner after inhibition of ribosome synthesis (Zaugg et al., 2011). Our findings suggest that inhibition of ribosome synthesis leads to a p53-dependent transcriptional program similar to what is observed during energetic stress.

Induction of autophagy by p53 has been shown to occur by different processes, and one of the best-understood mechanisms is inhibition of the mTORC1 pathway, a major negative regulator of autophagy. Nuclear p53 can also promote autophagy by transcriptionally activating target genes directly involved in promoting this process, such as LKB1, ULK1/2, ATG4, ATG7, and ATG10 (Kenzelmann Broz et al., 2013). Our findings suggest that activation of autophagy in response to nucleolar stress is mediated by the p53-dependent transcriptional up-regulation of SESTRIN 2, which inhibits mTORC1 activity through AMPK stimulation or by impairing mTORC1 localization to lysosomes (Parmigiani et al., 2014; Kim et al., 2015a, 2015b). Although not characterized as a p53 direct target, LC3B (*MAP1LC3B*, microtubule-associated protein 1 light-chain 3 transcript), required for elongation and maturation of the autophagosome, was also up-regulated in a p53-dependent manner after inhibition of ribosome biogenesis. This gene may represent a putative novel direct p53 target; however, further testing for p53 binding to the *MAP1LC3B* promoter region after nucleolar stress will be required to confirm this.

with bafilomycin A1 and CX-5461 dramatically impaired cell survival after only 24 h (Figure 7C). Similarly, chloroquine significantly sensitized cells to CX-5461 treatment (Figure 7D). We also observed a similar trend in cell sensitization in two other p53 positive cell lines (MCF7 and U2OS), where CX-5461 also up-regulated the expression of *CNKN1A* (p21^CIP1), *SESN2* (SESTRIN 2), *MAP1LC3B* (LC3B), and *CCNG2* (cyclin G2) (Supplemental Figure S3).

As our data indicate that the up-regulation of autophagy genes in response to nucleolar stress is p53-dependent (Figure 6), we next tested the effect of inhibiting autophagy on the sensitization of HCT 116 p53 −/− cells to CX-5461 treatment. Compared with p53 WT cells, HCT 116 p53 −/− cells showed a greater reduction in cell viability after treatment with bafilomycin A1 alone (Figure 7E). However, cotreatment of bafilomycin A1 with CX-5461 did not further decrease cell survival, contrary to what was observed for p53 WT cells (Figure 7E). Similarly, chloroquine did not show any dose-dependent sensitization to CX-5461 treatment (Figure 7F). Both bafilomycin A1 and chloroquine have been shown to also reduce mTORC1 activity by acting as lysosomal storage disorders mimetics, which could explain their effects on p53 −/− cell viability (Fedele and Proud, 2020). Together, these observations suggest that blocking autophagy could be used as a strategy to impair the survival of p53-positive cancer cells after inhibition of ribosome biogenesis.

Several drugs used for cancer treatment have been shown to induce autophagy in tumors as a response mechanism against cellular stress (Thorburn et al., 2014). Survival through induction
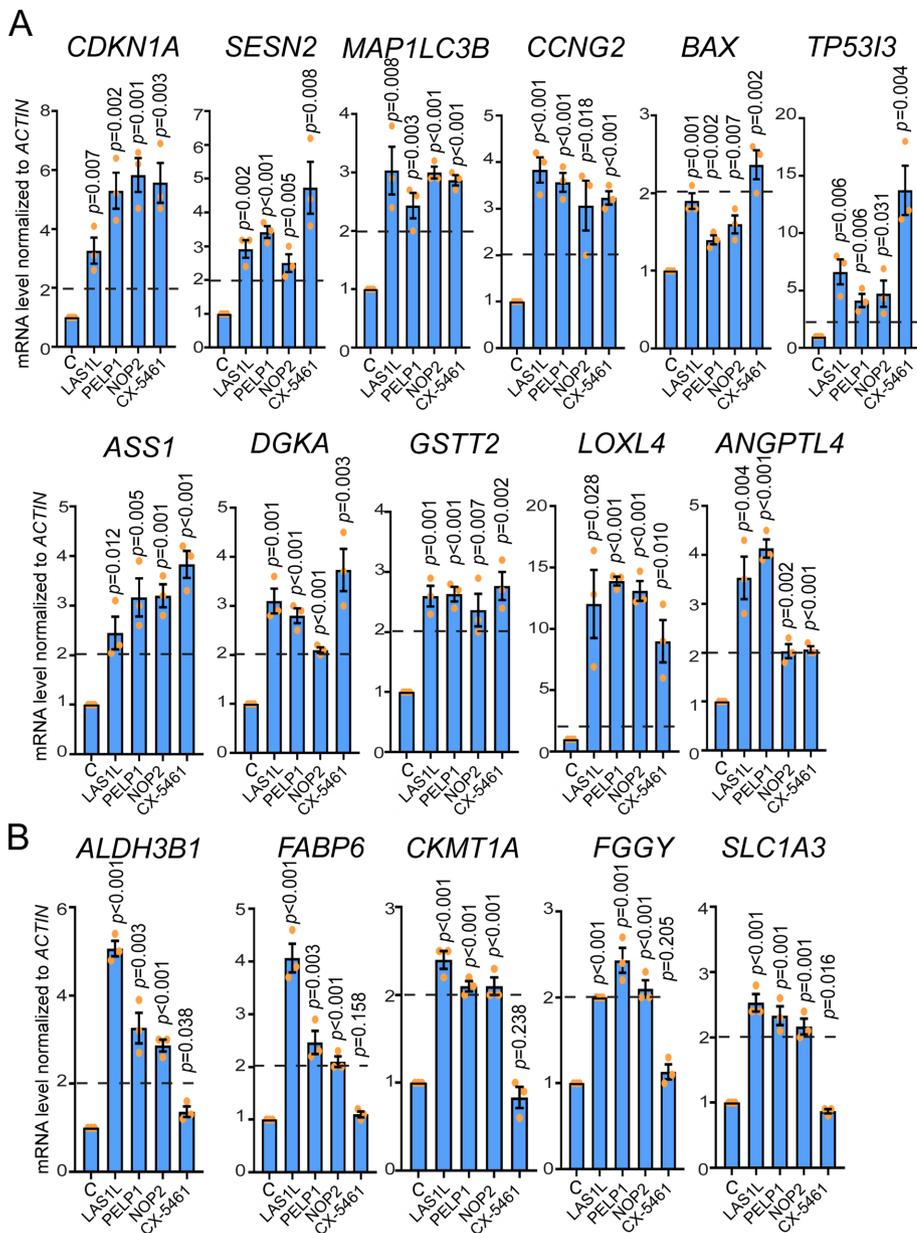
FIGURE 5: Q-RT-PCR validates the induction of mRNA transcripts after nucleolar stress. (A, B) Total RNAs from HCT116 cells treated with nontargeting control (C), LAS1L siRNA #1, PELP1 siRNA, NOP2 siRNA, DMSO, or 500 nM CX-5461 were analyzed 72 h after (for siRNA treatment) or 24 h after (CX-5461) by qRT-PCR to assess changes in gene expression. mRNA expression was normalized to *beta-ACTIN* values. Changes in gene expression are presented as fold change of siRNA-treated samples over control siRNA or CX-5461-treated samples over DMSO. A, genes that were confirmed to be up-regulated for all conditions. B, genes that were found to be up-regulated only in cells treated with LAS1L, PELP1, and NOP2 siRNA and not by CX-5461 treatment. The data are presented as means of three independent experiments ± SEM. Statistical significance comparing knockdowns with nontargeting control or CX-5461 to DMSO was calculated using two-tailed Student's *t* tests among biological replicates.

developing selective inhibitors that block or disassemble the Pol I transcription machinery. Although it also exerts a cytotoxic effect as a G-quadruplex stabilizer and topoisomerase II poison (Xu *et al.*, 2017; Bruno *et al.*, 2020), the selective Pol I inhibitor CX-5461 is currently in clinical trial for the treatment of breast and advanced hematologic cancers. Studies have suggested that CX-5461 induces autophagy via inhibition of mTOR signaling in osteosarcoma and leukemia cells (Li *et al.*, 2016; Okamoto *et al.*, 2020). Our data corroborate these findings and suggest that inhibiting autophagy may be a viable strategy to heighten the responsiveness of ribosome biogenesis-targeted therapies, at least in p53 WT tumors. The majority of p53 mutations found in cancers are missense substitutions leading to the expression of mutant proteins with unique protumorigenic gain-of-function activities (Muller and Vousden, 2013). Interestingly, mutant p53 proteins have been shown to have the ability to hypertransactivate p53-target genes, such as *CDKN1A* (p21[CIP1]) and *SESN2* (SESTRIN 2), after metabolic stress, thus promoting cell cycle arrest and cell survival (Tran *et al.*, 2017). It will be important to evaluate the transcriptional program activated by these mutant p53 proteins after nucleolar stress to determine whether inhibition of rRNA synthesis would beneficially synergize with concomitant inhibition of autophagy or metabolic processes in the treatment of tumors harboring gain-of-function p53 mutations.

MATERIALS AND METHODS
Request a protocol through *Bio-protocol*.

Cell culture, RNA interference, and drug treatments
Cells (HCT116 WT, HCT116 p53 –/–, MCF7, and U2OS) were cultured at 37°C with 5% CO_2 in DMEM high glucose (Hyclone) supplemented with 5% fetal bovine serum (FBS, Hyclone) and penicillin–streptomycin. All cells used were obtained from ATCC, which performs short-tandem repeat profiling to confirm the identity of their cell lines. Cell lines are tested for mycoplasma contamination every 6 months by PCR assay with appropriate positive controls (Young *et al.*, 2010). Synthetic short interfering RNA (siRNA) oligonucleotides (Sigma) were delivered into cells with Lipofectamine RNAi Max (Invitrogen). siRNA oligonucleotides (40 nM) were transfected using the reverse transfection protocol according to the manufacturer's instructions. The following siRNA oligonucleotide targeting sequences were used:

of autophagy is often considered an underlying cause of therapeutic resistance in multiple cancer types. Thus, there has been a growing interest in developing clinical trials aiming at blocking autophagy to potentiate the effect of a cancer drug (Levy *et al.*, 2017). Targeting rRNA production has emerged as a promising therapeutic for cancer and much of the efforts have focused on

FIGURE 6: p53-dependent gene expression after nucleolar stress. HCT116 or HCT116 p53 –/– cells were treated with nontargeting control, LAS1L siRNA #1, DMSO, or 500 nM CX-5461. Seventy-two h after (for siRNA treatment) of 24 h after (CX-5461), total RNA was extracted and analyzed by qRT-PCR to assess changes in gene expression. mRNA expression was normalized to *beta-ACTIN* values. Changes in gene expression are presented as fold change of LAS1L siRNA over control siRNA or CX-5461 over DMSO. The data are presented as means of three independent experiments ± SEM. Statistical significance comparing knockdowns with nontargeting control or CX-5461 to DMSO was calculated using two-tailed Student's *t* tests among biological replicates.

CX-5461 (Cellagen Technologies) was reconstituted in DMSO and used at a final concentration of 500 nM. Bafilomycin A1 (Sigma) was reconstituted in DMSO and used at final concentrations of 50 and 100 nM. Chloroquine (Sigma) was reconstituted in sterile dH2O and used at final concentrations of 5 and 10 µM.

RNA, microarray, genes set enrichment analysis, and quantitative RT-PCR analysis

HCT116 cells were treated with control siRNA (in duplicate), LAS1L siRNA #1, or LAS1L siRNA #2 for 72 h. Total RNAs, extracted with Trizol reagent (Invitrogen), were amplified, labeled, and hybridized to an Illumina HumanHT-12 v4 expression bead chip kit using standard protocols at the Microarray Core Lab, UT McGovern Medical School. All analyses were performed using GenomeStudio software (Illumina). Genes set enrichment analysis (GSEA) was performed separately on up-regulated and down-regulated genes using the ontology and canonical pathways gene sets. The *p* value from the hypergeometric distribution for k-1, K, N-K, and n, where k is the number of genes in the intersection of the query set with a set from MSigDB (Subramanian *et al.*, 2005; Liberzon *et al.*, 2011, 2015), K is the number of genes in the set from MSigDB, N is the total number of genes in the universe (all known human gene symbols), and n is the number of genes in the query set, is represented. A cut off for FDR *q*-value less than 0.05 was selected. Only the top 10 gene sets were considered.

For quantitative RT-PCR analysis, complementary DNA (cDNA) was synthesized using 1 µg of total RNA with the QuantiTect reverse transcription kit (Qiagen). Real-time PCR analysis was performed on a Light Cycler 480 real-time PCR instrument. Values were normalized to *beta-ACTIN* mRNA. A qPCR standard curve was performed to test the efficiency of each primer pairs. The primers used for the quantitative PCR are listed in Supplemental Table S3.

nontargeting scramble: 5'-GAUCAUACGUGCGAUCAGAdTdT-3';
human LAS1L #1: 5'-CUGAUACGCUGUAAGCUCUdTdT-3';
human LAS1L #2: 5'-CAUUUAUACCCAGAGUGGAdTdT-3';
human PELP1 5'-GGAAUGAAGGCUUGUAUGAdTdT-3';
human NOP2 5'-CACCUGUUCUAUCACAGUAdTdT-3';
human SESTRIN 2 #1 5'-CCUACAAUACCAUCGCCAUdTdT-3';
human SESTRIN 2 #2 5'-CGAAGAAUGUACAACCUCUdTdT-3';
human ATG7 #1 5'-CAGCUAUUGGAACACUGUAdTdT-3';
human ATG7 #2 5'-GAGAUAUGGGAAUCCAUAAdTdT-3';
human RPL11/uL5 5'-GGUGCGGGAGUAUGAGUUAdTdT-3'

Flow cytometry and cell survival assays

Cell cycle progression was assayed by DNA content using propidium iodide (PI) and BrdU labeling (anti-BrdU: BD Biosciences, Cat# 347580, goat anti-mouse Alexa-Fluo488, Invitrogen, Cat# A11001) followed by flow cytometry analysis as described previously (Castle *et al.*, 2010). Analyses were performed on a BD LSRFortessa. For the survival assay, cells were transfected with nontargeting control, SESTRIN 2, or ATG7 siRNAs for 3 d. On day 2 posttransfections, cells were treated with vehicle (dimethyl sulfoxide, DMSO) or CX-5461 (500 nM) for 24 h. Another set of cells were grown in a monolayer and treated for 24 h with DMSO or CX-5461 (500 nM) in conjunction with either

FIGURE 7: Inhibition of autophagy sensitizes cancer cells to nucleolar stress. (A) HCT116 cells were treated with nontargeting control (C), SESN2 #1, SESN2 #2, ATG7 #1, or ATG7 #2 siRNAs. Seventy-two h later, cells were collected and knockdowns were monitored by Western blotting. (B) Cells from panel A were treated with DMSO or 500 nM CX-5461 for 24 h. HCT116 (C, D) or HCT116 p53 –/– cells were treated with vehicle (DMSO) or 500 nM CX-5461 for 24 h in the presence of bafilomycin (0, 50 nM, 100 nM; C, E) or chloroquine (0, 5 μM, 10 μM; D, F). Cell viability, B–F, was determined by trypan blue exclusion. The data are presented as means of four, E, F, or three, B–D, independent experiments ± SEM. Statistical significance comparing CX-5461 to DMSO was calculated using two-tailed Student's *t* tests among biological replicates.

by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad). The following antibodies were used: LAS1L (Cat#: A304-438A, Bethyl Laboratories), p53 (Cat#: SC-126, Santa Cruz Biotechnology), p21[CIP1] (Cat#: 556430, BD Biosciences), β-ACTIN (Cat#: SC-69879, Santa Cruz Biotechnology), GAPDH (Cat#: 47724, Santa Cruz Biotechnology); RPL11/uL5 (Cat#: SAB1402896, Sigma), LC3B (Cat#: 3868, Cell Signaling), PELP1 (Cat#: A300-180A, Bethyl Laboratories), NOP2 (Cat#: A302-018A, Bethyl Laboratories), Cyclin E (Cat#: SC-198, Santa Cruz Biotechnology), SESTRIN 2 (Cat#: 10795-1-AP, Proteintech), and ATG7 (Cat#: SC-376212, Santa Cruz Biotechnology).

Fluorescence microscopy

For the detection of LAS1L by immunofluorescence, cells grown on cover glass were fixed with 4% paraformaldehyde at room temperature for 15 min. Cells were then permeabilized at room temperature with 0.1% Triton for 10 min. All samples were blocked with 1% BSA for 30 min, washed with PBS, and incubated with an anti-LAS1L antibody (Cat#: AV34629, Sigma) for 1 h. Cells were washed with PBS containing 0.05% Tween-20 and incubated with secondary antibody for 1 h (Alexa-Fluor 488 goat anti-rabbit antibody, Cat#: A11001, Invitrogen). Cells were washed again with PBS containing 0.05% Tween-20, stained with DAPI (Molecular Probes), and mounted on slides with Vectashield (Vector Labs). To visualize LC3 localization to autophagosomes, HCT116 cells were transfected with a plasmid expressing pEGFP-LC3 (a gift from Toren Finkel (Addgene plasmid #24920; Lee *et al.*, 2008) and pools of stable clones were established after selection with G418. Fluorescence microscopy was performed on a Zeiss Axioskop 40 fluorescence microscope with a Plan-APOCHROMAT 63×/1.4 NA oil DIC objective. Images were acquired with an Axiocam MRm camera using the Axiovision

bafilomycin A1 (0, 50 nM, or 100 nM) or chloroquine (0, 5 μM, or 10 μM). Cell viability for both sets of experiments was determined by trypan blue exclusion.

Immunoblotting and antibodies

For immunoblotting, cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) plus protease inhibitors (aprotinin, leupeptin, AEBSF) and phosphatase inhibitor cocktail (ThermoFisher) for 15 min on ice. Lysates were cleared by centrifugation at 22,000 × *g* for 10 min at 4°C. Protein concentrations were evaluated with a BCA kit (Pierce). Proteins were separated

Release 4.6 software with a capturing resolution of 1388 × 1040 pixels. All microscopy was performed at room temperature, and all images were prepared in Adobe Photoshop and Adobe Illustrator.

Statistical analyses

Results are represented as mean ± SEM (standard error of the mean) except as otherwise specified. Differences between groups were considered significant at *p* < 0.05 by a two-tailed Student's *t* test. Data shown are average values of three independent experiments except as otherwise specified (each qPCR sample was performed with technical triplicates).

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