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Inhibition of Nonsense-Mediated RNA Decay Activates Autophagy

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Nonsense-mediated RNA decay (NMD) is an mRNA surveillance mechanism which rapidly degrades select cytoplasmic mRNAs. We and others have shown that NMD is a dynamically regulated process inhibited by amino acid deprivation, hypoxia, and other cellular stresses commonly generated by the tumor microenvironment. This inhibition of NMD can result in the accumulation of misfolded, mutated, and aggregated proteins, but how cells adapt to these aberrant proteins is unknown. Here we demonstrate that the inhibition of NMD activates autophagy, an established protein surveillance mechanism, both *in vitro* and *in vivo*. Conversely, the hyperactivation of NMD blunts the induction of autophagy in response to a variety of cellular stresses. The regulation of autophagy by NMD is due, in part, to stabilization of the documented NMD target ATF4. NMD inhibition increases intracellular amino acids, a hallmark of autophagy, and the concomitant inhibition of autophagy and NMD, either molecularly or pharmacologically, leads to synergistic cell death. Together these studies indicate that autophagy is an adaptive response to NMD inhibition and uncover a novel relationship between an mRNA surveillance system and a protein surveillance system, with important implications for the treatment of cancer.

Nonsense-mediated RNA decay (NMD) is a cellular surveillance system that rapidly degrades select mRNAs. While the exact mechanism of NMD is still not completely delineated, current models suggest that during a pioneer round of translation, the translation complex pauses at premature termination codons (PTCs) upstream of exon junction complexes (EJCs). Crucial components of the NMD mechanism, including Upf1/Rent1 and Upf2/Rent2, bridge the EJC to a paused translation complex and initiate RNA degradation. In addition to PTCs located upstream of EJCs, long 3′ untranslated regions and other, less-well-defined features of the processed mRNA transcript can result in mRNA degradation by NMD (reviewed in reference 1). Up to 30% of all mutations responsible for human genetic disorders, including cystic fibrosis, muscular dystrophy, and thalassemia, result in PTCs upstream of EJCs (2). NMD also degrades nonmutated mRNAs, including those important for the response to cellular stress and amino acid transport (3–5). The recent observation that NMD is inhibited by amino acid deprivation, hypoxia, the accumulation of unfolded proteins in the endoplasmic reticulum (ER), and other cellular stresses that lead to the phosphorylation of eukaryotic initiation factor 2α (eIF2α) has led to a greater appreciation of the potential role that the dynamic regulation of gene expression by NMD could play in physiology and pathology (3–5).

The stress-induced inhibition of NMD stabilizes the stress-responsive transcription factor ATF4, augments the ER stress response, and permits cells to grow in soft agar and as tumor explants, conditions in which cells are deprived of nutrients and oxygen (5). However, the full biological consequences of NMD regulation are unknown. These consequences include the stabilization of mutated and alternatively spliced transcripts which could encode deleterious dominant negative, truncated, and/or misfolded proteins (6–10). The recognition that NMD is dynamically regulated raises the question of how the cell adapts to these aberrant proteins when NMD is physiologically or pathologically inhibited. Autophagy, a process in which misfolded/aggregated cytoplasmic proteins are enveloped within autophagosomes which later fuse to digestive lysosomes, serves both as a cellular surveillance mechanism to rid the cell of detrimental proteins and as a source of amino acids during metabolic stress (reviewed in reference 11). However, the relationship between NMD activity and autophagy has not been examined.

Many of the cellular stresses which inhibit NMD, including amino acid deprivation, hypoxia, and viral infection, also promote autophagy. In fact, eIF2α phosphorylation not only plays a central role in the stress-induced inhibition of NMD but also is necessary for the induction of autophagy during amino acid deprivation (12, 13). We thus hypothesized that the inhibition of NMD plays an important role in the induction of autophagy. Specifically, we reasoned that autophagy would be activated by NMD inhibition. Furthermore, we theorized that the activation of autophagy would enable the cell to dispose of mutated, misfolded, and aggregated proteins that accumulate with the physiological inhibition of NMD and thus would serve as an adaptive response to NMD inhibition.

MATERIALS AND METHODS

Cell culture. Cells were cultured as previously described (3) and treated with 2.5 mg/ml tunicamycin, 300 nM rapamycin, or 60 μM chloroquine or were depleted of amino acids by culturing in Krebs-Ringer bicarbonate buffer (10 mM glucose, 0.5 mM $MgCl_2$, 0.45 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO4, 1.5 mM NaH_2PO_4, 15 mM $NaHCO_3$) supplemented with 10% dialyzed fetal bovine serum. The acquisition, culture, and deple-

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tion and overexpression of Upf1/Rent1 and Upf2/Rent2 retroviruses have been previously reported (3, 5). The LKO.1 shATF4 virus (TRCN0000013573) was obtained from Sigma, and a green fluorescent protein (GFP)-LC3 fusion, a kind gift from G. Kroemer, was cloned into the retroviral vector pQXCIN or pBABE and used to infect U2OS, HCT116, and mouse embryo fibroblast (MEF) cells using standard retroviral generation and infection techniques (3, 5).

Immunoblotting and real-time PCR. Immunoblotting was done using fluorescent secondary antibodies, and blots were quantitated with Li-Cor reagents as previously described (5). Membranes were stained with antibodies directed against LC3 (Novus; NB600-1384), p62 (Enzo; PW9860), SLC7A11 (Novus; NB300-318), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling; 5625). Real-time PCR and determination of mRNA stability in cells treated with the RNA synthesis inhibitor 5,6-dichlorobenzimidazole-1-β-D-ribofuranoside (DRB) (Sigma) were performed as previously reported with SLC7A11 and SLC38A2 primers (sequences are available upon request) (3, 5).

Microscopy and assessment of LC3 foci. Cells were fixed with 3.7% paraformaldehyde for 20 min and stained with LC3 antibody with secondary stain. Both these foci and GFP foci were visualized with confocal microscopy as previously described (3). GFP-LC3-expressing cells containing three or more foci were counted as positive and quantified as a proportion of all cells within a given field. Endogenous LC3 foci were quantified using the ImageJ software. Image intensity thresholds were set to a constant value to include particles with a high signal compared to background. Regions of interest were drawn around each cell in a field, and the number of foci were counted using the "analyze particles" function. Mice expressing the dominant negative Upf1/Rent1 and control mice have been previously described (14). Paraffin-embedded thymus and spleen sections were deparaffinized, followed by antigen retrieval in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) at 95°C for 20 min. Slides were then stained with LC3 antibody with secondary stain. Electron microscopy (EM) was done by the NYU Microscopy Core with a Philips CM200 cryoelectron microscope equipped with Gatan UltraScan 1000 2kx2k charge-coupled device (CCD) camera.

Amino acid quantitation. Control U2OS cells and U2OS cells overexpressing Upf1/Rent1 or depleted of either Upf1/Rent1 or UPF2/Rent2, in 6 replicates, were extracted and split for gas chromatography-mass spectrometry (MS) or liquid chromatography-MS platforms by Metabolon (Durham, NC), and the profiles of over 1,000 metabolites were assessed.

Amino acid transporter assay. Equal number of either U2OS or HeLa control cells and Upf1/Rent1 and Upf2/Rent2 knockdown U2OS or HeLa cells were seeded 24 h prior to the experiment. Cells were rinsed twice with Krebs-Ringer HEPES (pH 7.4) and were incubated in buffered Krebs-Ringer solution containing 1μCi of [^{14}C]methylaminoisobutyric acid ([^{14}C]MeAIB) at 37°C for 2.5 min and 5 min. After the indicated incubations, cells were washed twice with buffer and lysed in 1% SDS. Radioactivity was measured using a liquid scintillation counter (Perkin-Elmer, Inc.), and counts were normalized with protein concentration.

Cell viability assay. Control HCT116 cells, Upf1/Rent1 and Upf2/Rent2 knockdown cells, or Upf1/Rent1-overexpressing HCT116 cells were treated with chloroquine for the indicated times. The control HCT116 cells were treated with NMD inhibitor alone, chloroquine alone, or both drugs combined for 24 and 48 h. After the above-mentioned treatments, cells and medium were collected and spun at 1,000 rpm for 5 min. The cell pellets were stained with trypan blue, and dead cells were measured by using a Countess automated cell counter (Invitrogen).

RESULTS

NMD inhibition activates autophagy. To explore the relationship between NMD and autophagy, we first suppressed NMD activity in a variety of tractable cell lines with validated Upf1/Rent1 or Upf2/Rent2 short hairpin RNAs (shRNAs). We and others have demonstrated that the modest depletion of either of these critical NMD components is sufficient to inhibit NMD, leading to both the formation of alternatively spliced mRNAs and the upregulation and stabilization of approximately 700 mRNAs which significantly overlap those mRNAs stabilized with the chemical or physiological inhibition of NMD (4–6). Autophagy is a dynamic and complex process, and to reliably assess its activity, we utilized multiple complementary assays, including immunoblotting to evaluate the formation of the faster-migrating LC3II from LC3I that occurs during autophagosome formation and the expression of p62 that is degraded in autophagosomes, indirect and direct immunofluorescence to examine the migration of endogenous LC3 and LC3-GFP from a diffuse cytoplasmic distribution to punctate intracellular foci, which reflects autophagosome formation, and electron microscopy to directly survey the formation of autophagosomes. We also relied on the robust and validated assay in which an increase in autophagy is reflected by the generation of free GFP from an LC3-GFP fusion protein that occurs in autophagosomes (15). Because not all autophagy assays are informative in every cell line (16), we assessed autophagy in multiple NMD-inhibited cell lines (see Fig. S1 in the supplemental material).

The inhibition of NMD by Upf1/Rent1 or Upf2/Rent2 depletion (40% and 50%, respectively) in the osteosarcoma cell line U2OS led to a consistent modest induction of autophagy, as noted by increased LC31-to-LC3II conversion and free GFP formation (Fig. 1A, compare lane 1 to lanes 5 and 9). When autophagy was provoked in these cells with the mTORC1 inhibitor rapamycin, Upf1/Rent1 and Upf2/Rent2 depletion augmented autophagy, with increased LC3I-to-LC3II conversion and free GFP generation noted in the NMD-inhibited cells compared to control cells (Fig. 1A, compare lane 2 to lanes 6 and 10). Consistent with these findings, when autophagy was induced with rapamycin treatment or amino acid deprivation, the depletion of Upf1/Rent1 or Upf2/Rent2 led to increased punctate GFP-LC3 foci compared to those in control U2OS cells (Fig. 1B). We also noted increased endogenous LC3 foci in Upf1/Rent1- and Upf2/Rent2-depleted HCT116 cells compared to control cells, both at baseline and upon rapamycin treatment (Fig. 1C). The formation of autophagosomes is the gold standard for autophagy, and we noted that Upf1/Rent1 depletion in U2OS cells also led to double-membraned vesicles, consistent with autophagosomes (Fig. 1D).

Autophagy is a dynamic process, and increased LC3II and autophagosome formation with NMD inhibition could reflect either increased autophagy or decreased fusion of autophagosomes with functional lysosomes. Although the appearance of the generation of free GFP from LC3-GFP fusions suggests that the inhibition of NMD increases the delivery of cargo to functional lysosomes, as an additional method to differentiate between these two possibilities, we assessed the role of NMD inhibition in autophagy in the presence of chloroquine, an inhibitor of late autophagy that blocks the acidification of lysosomes. Even in the presence of chloroquine, we noted that Upf1/Rent1 or Upf2/Rent2 depletion in U2OS cells increased LC3I-to-LC3II conversion and free GFP generation (Fig. 1A, compare lanes 3 and 4 to lanes 7, 8, 11, and 12). As another indicator that the increase in autophagy markers with NMD inhibition is due to increased true autophagic flux, we noted that the augmentation of autophagy with NMD inhibition led to decreased p62 expression, which occurs when autophagosomes fuse with lysosomes, with and without rapamycin treatment in HeLa cells (Fig. 1E) and in nontransformed mouse embryo fibroblasts (MEFs) (Fig. 1F).

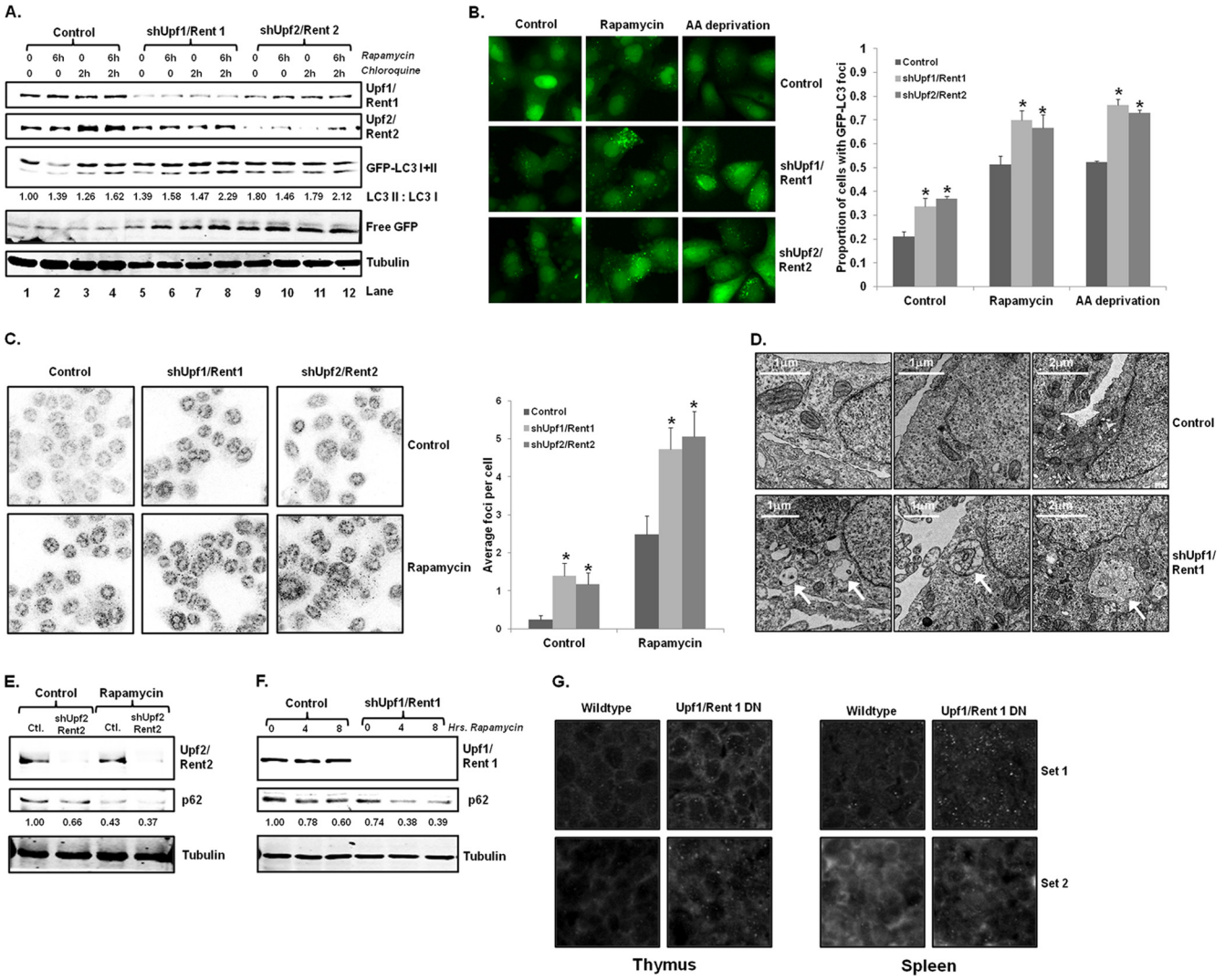

FIG 1 Inhibition of NMD activates autophagy. (A) U2OS cells stably expressing a GFP-LC3 fusion and expressing either a control, shUpf1/Rent1, or shUpf2/Rent2 lentivirus were treated with rapamycin for 6 h, with or without chloroquine, and protein lysates were immunoblotted for GFP and other noted proteins. (B) These same cells were treated with rapamycin for 2 h, amino acid deprivation for 8 h, or tunicamycin for 8 h, and LC3 foci were visualized (left panel) and quantitated (right panel) by confocal microscopy as described in Materials and Methods. *, $P < 0.05$ by Student's *t* test compared to a similarly treated control. (C) Control, Upf1/Rent1, and Upf2/Rent2 HCT116 cells were treated with rapamycin for 8 h, and endogenous LC3 foci were visualized (left panel) and quantitated (right panel). (D) Control and Upf1/Rent1-depleted U2OS cells were examined for autophagosomes by EM. (E and F) HeLa cells with Upf1/Rent1 depletion (E) and MEFs with Upf2/Rent2 depletion (F) were treated with rapamycin and assessed for p62 expression. (G) Mice expressing a dominant negative Upf1/Rent1 ($n = 2$) or littermate controls ($n = 2$) had thymi and spleens removed at fetal day 17 and assessed for LC3 as described in Materials and Methods.

We next sought to confirm our findings *in vivo*. During lymphocyte development, T-cell receptor (TCR) and immunoglobulin genes undergo a series of programmed rearrangements, two-thirds of which result in a PTC. Depletion of Upf2/Rent2 in hematopoietic cells leads to the accumulation of nonproductive rearranged by-products from the T-cell receptor locus, which is detrimental to developing T cells (17). Similarly, the ubiquitous expression of a dominant negative form of Upf1/Rent1 results in a crisis in thymus development and thymocyte apoptosis coincident with the onset of TCR β allele rearrangement by fetal day 19, and it also impairs early B-cell development coincident with the timing of nonsense transcript production (14). When we examined the thymi and spleens of mice expressing a dominant negative Upf1/Rent1 at fetal day 17 in multiple independent founders, we observed a marked increased in punctated LC3 foci compared to those in littermate controls (Fig. 1G), suggesting that the inhibition of NMD increases autophagy *in vivo*. Thus, using a range of complementary assays in diverse cell lines depleted of either Upf1/Rent1 or Upf2/Rent2, as well as a model of *in vivo* NMD suppression, our findings indicate that the inhibition of NMD augments autophagy.

Activation of NMD decreases autophagy. Since the inhibition of NMD increases autophagy, we next determined whether the activation of NMD suppresses autophagy. We and others have noted that overexpression of Upf1/Rent1 is sufficient to hyperactivate NMD and increase the degradation of multiple NMD-targeted transcripts (3, 5, 18). The overexpression of Upf1/Rent1 in U2OS cells led to a reproducible decrease in LC3II and free GFP

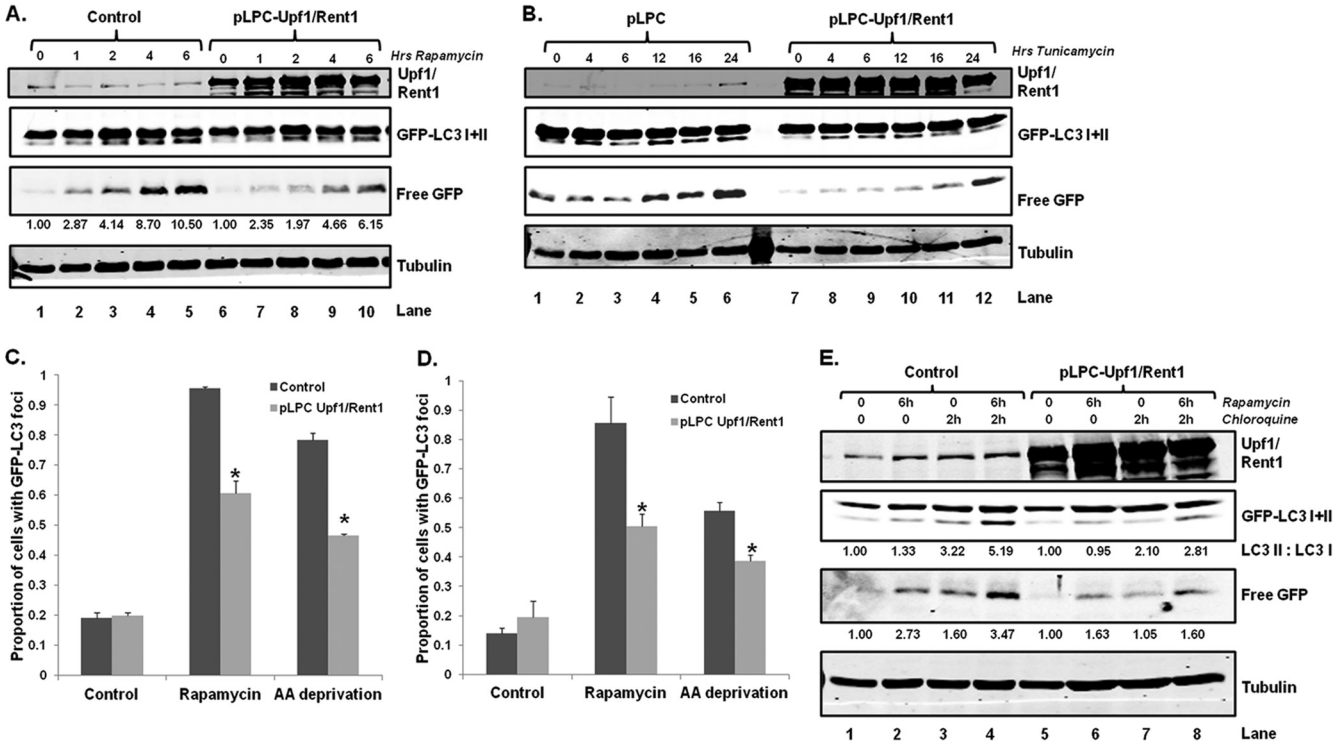

FIG 2 Hyperactivation of NMD blunts stress-induced autophagy. (A and B) U2OS cells stably expressing a GFP-LC3 fusion were treated with rapamycin (A) or tunicamycin (B) for the indicated time periods and immunoblotted for GFP and other noted proteins. (C and D) These cells (C) and MEFs (D) expressing GFP-LC3 were treated with rapamycin for 8 h or deprived of amino acids for 36 h, and LC3 foci were quantitated. *, $P < 0.05$ by Student's *t* test compared to a similarly treated control. (E) U2OS cells expressing GFP-LC3 were treated with rapamycin with and without chloroquine and immunoblotted for GFP and other noted proteins.

generation (Fig. 2A [compare lanes 1 to 6] and B [compare lanes 1 to 7]). The blunting of autophagy with hyperactivation of NMD was more dramatic when autophagy was induced by rapamycin (Fig. 2A) or tunicamycin (Fig. 2B) treatment. The overexpression of Upf1/Rent1 also led to decreases in LC3 foci in U2OS cells (Fig. 2C) and MEFs (Fig. 2D) stably expressing GFP-LC3. When the acidification of autophagosomes was inhibited by the addition of chloroquine, LC3I-to-LC3II conversion and the generation of free GFP were still diminished in U2OS cells overexpressing Upf1/Rent1 compared to control cells (Fig. 2E, compare lane 4 to lane 8), consistent with a decrease in the autophagic flux with the hyperactivation of NMD.

ATF4 is responsible, in part, for NMD regulation of autophagy. We next pursued the mechanism underlying the relationship between NMD activity and autophagy. The regulation of autophagy is complex, and although mTOR activity is a key regulator of autophagy we did not observe alterations of 4EBP1 or S6 kinase phosphorylation, markers of mTOR activity, with NMD inhibition or hyperactivation in a variety of cell lines (see Fig. S2 in the supplemental material). Another mechanism by which NMD could regulate autophagy is by targeting mRNAs involved in autophagy. The direct NMD target ATF4 has been implicated in autophagy by its ability to directly transactivate the essential autophagy genes encoding microtubule-associated protein 1 light chain 3 beta (LC3B), an important component of the autophagosome membrane, and ATG5, an E2 ubiquitin ligase which is necessary for autophagy due to its role in autophagosome elongation (3–5, 13, 17). Consistent with the role of ATF4 in autophagy, the efficient depletion of ATF4 in U2OS cells (as demonstrated by minimal ATF4 induction in response to tunicamycin treatment) blunted both free GFP formation and LC3I-to-LC3II conversion with rapamycin or tunicamycin treatment compared to those in control cells (Fig. 3A, compare lanes 2 to 8 and lanes 3 to 9).

We then assessed the effect of Upf2/Rent2 depletion on autophagy in cells that were also depleted of ATF4. As we previously observed, Upf2/Rent2 depletion alone increased free GFP generation (Fig. 3, compare lanes 1 to 4, lanes 2 to 5, and lanes 3 to 6). However, we found that the conversion and free GFP generation that we observed with Upf2/Rent2 depletion was dramatically blunted with the concurrent depletion of ATF4 (Fig. 3A, compare lanes 4 to 10, lanes 5 to 11, and lanes 6 to 12). Similarly, the increase in LC3 foci with NMD inhibition was significantly decreased when ATF4 was also depleted (Fig. 3B). Although ATF4 was only minimally increased with Upf2/Rent2 depletion in this and other studies (Fig. 3A, compare lane 1 to 4) (5), the increase in ATG5 mRNA expression seen in Upf1/Rent1-depleted cells was blunted when ATF4 was also depleted (Fig. 3C). Although LC3B mRNA expression was not increased with simple Upf1/Rent1 depletion, it was increased when these cells were treated with rapamycin compared to in control cells treated with rapamycin; this increase in LC3B was also blunted when ATF4 was codepleted (Fig. 3C). Together these data suggest that the stabilization of ATF4 plays at least some role in the induction of autophagy with NMD inhibition, through the upregulation of ATG5 and LC3B, although there are likely contributions from other mechanisms.

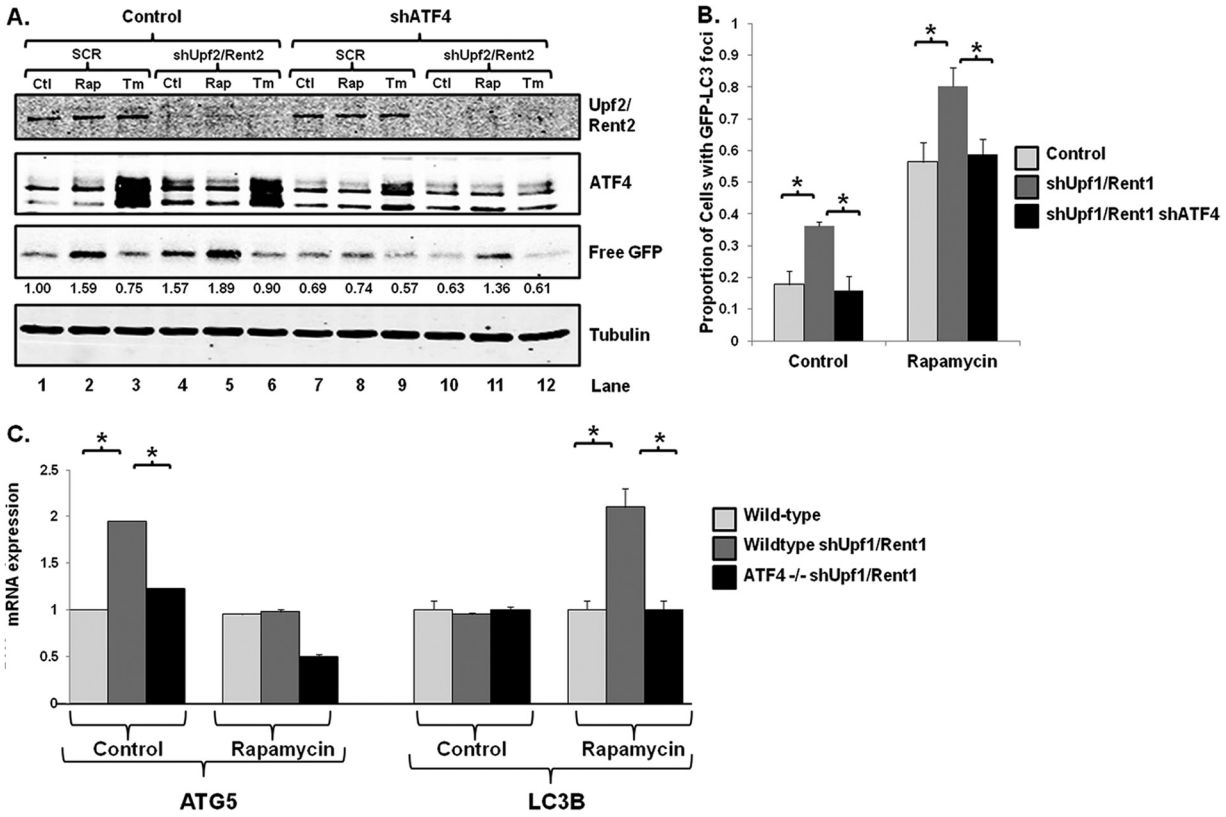

FIG 3 ATF4 contributes to the induction of autophagy during NMD inhibition. (A and B) U2OS cells stably expressing GFP-LC3 were depleted of ATF4, Upf2/Rent2, or both and treated with rapamycin or tunicamycin for 8 h, and GFP was assessed by immunoblotting (A) or LC3 foci (B). *, $P < 0.05$ by Student's *t* test compared to a similarly treated control. (C) Control and Upf1/Rent1- and ATF4-deficient MEFs were treated with vehicle or rapamycin for 8 h and assessed for ATG5 and LC3B mRNA expression. *, $P < 0.05$ by Student's *t* test.

Induction of autophagy with NMD inhibition increases intracellular amino acid concentrations. We next investigated the consequence of the induction of autophagy with NMD inhibition. One of the hallmarks of autophagy is the generation of free amino acids, which occurs as an adaptive response during periods of prolonged starvation (19–21). Of the 200 measurable intracellular metabolites, amino acids were the most consistently affected by NMD regulation. Specifically, NMD hyperactivation by Upf1/Rent1 overexpression (NMD-A) significantly ($P < 0.05$) decreased, and/or NMD inhibition with Upf1/Rent1 depletion (NMD-I1) or Upf2/Rent2 depletion (NMD-I2) significantly increased, the intracellular concentrations of glycine, serine, alanine, aspartate, glutamate, lysine, isoleucine, and valine (Fig. 4A). In other cases, although alterations were not significant, the levels of specific amino acids tended to be lower with NMD activation and higher with NMD inhibition (see Table S1 in the supplemental material).

Increases in intracellular amino acids could be the result of increased autophagy or, alternatively, of a net increase of inward cellular transport. In fact, of the mRNAs found to be increased with the depletion of Upf1/Rent1, 15% encode proteins involved in amino acid transportation, and in addition, many amino acid transporters are upregulated by the NMD target ATF4 (4, 22). To determine the role that intracellular transport could be playing in increasing intracellular amino acid concentrations with NMD inhibition, we first examined the mRNA induction of 23 amino acid transporters with Upf1/Rent1 and Upf2/Rent2 depletion in U2OS and HeLa cells and MEFs. In accordance with previous reports, we found that many NMD transporter transcripts were upregulated by the depletion of Upf1/Rent1 or Upf2/Rent2 (see Table S2 in the supplemental material). While most were upregulated only in the presence of ATF4, four transcripts (SLC7A11, SLC38A2, SLC1A3, SLC7A11, and SLC3A2) were also upregulated in ATF4-deficient cells and/or stabilized with NMD inhibition, and these are thus direct NMD targets (Fig. 4B and C; see Tables S2 and S3 in the supplemental material). However, despite the observation that SLC3A2 mRNA is stabilized (Fig. 4B) and upregulated (Fig. 4C) by NMD inhibition, the expression of SLC3A2 protein was unaltered by NMD activity (Fig. 4D). Similarly, while many of the stresses that inhibit NMD led to the stabilization (Fig. 4E) and upregulation (Fig. 4F) of the SLC38A2 mRNA, the activity of the SLC38A2-encoded neutral amino acid transporter SNAT2, which is responsible for transporting many of the amino acids noted to be increased in NMD-inhibited cells (including glycine, serine, alanine, lysine, isoleucine, and valine), as measured by the intracellular transport of the nonmetabolized amino acid analogue MeIAB, was decreased, not increased, in HeLa and U2OS cells depleted of Upf1/Rent1 or Upf2/Rent2, (Fig. 4G). Thus, although SLC3A2 and SLC38A2 mRNAs are direct NMD targets, their protein and functional activities are not regulated by NMD. Although we did not assess the protein expression and function of all amino acid transporters with NMD inhibition, our data are most consis-

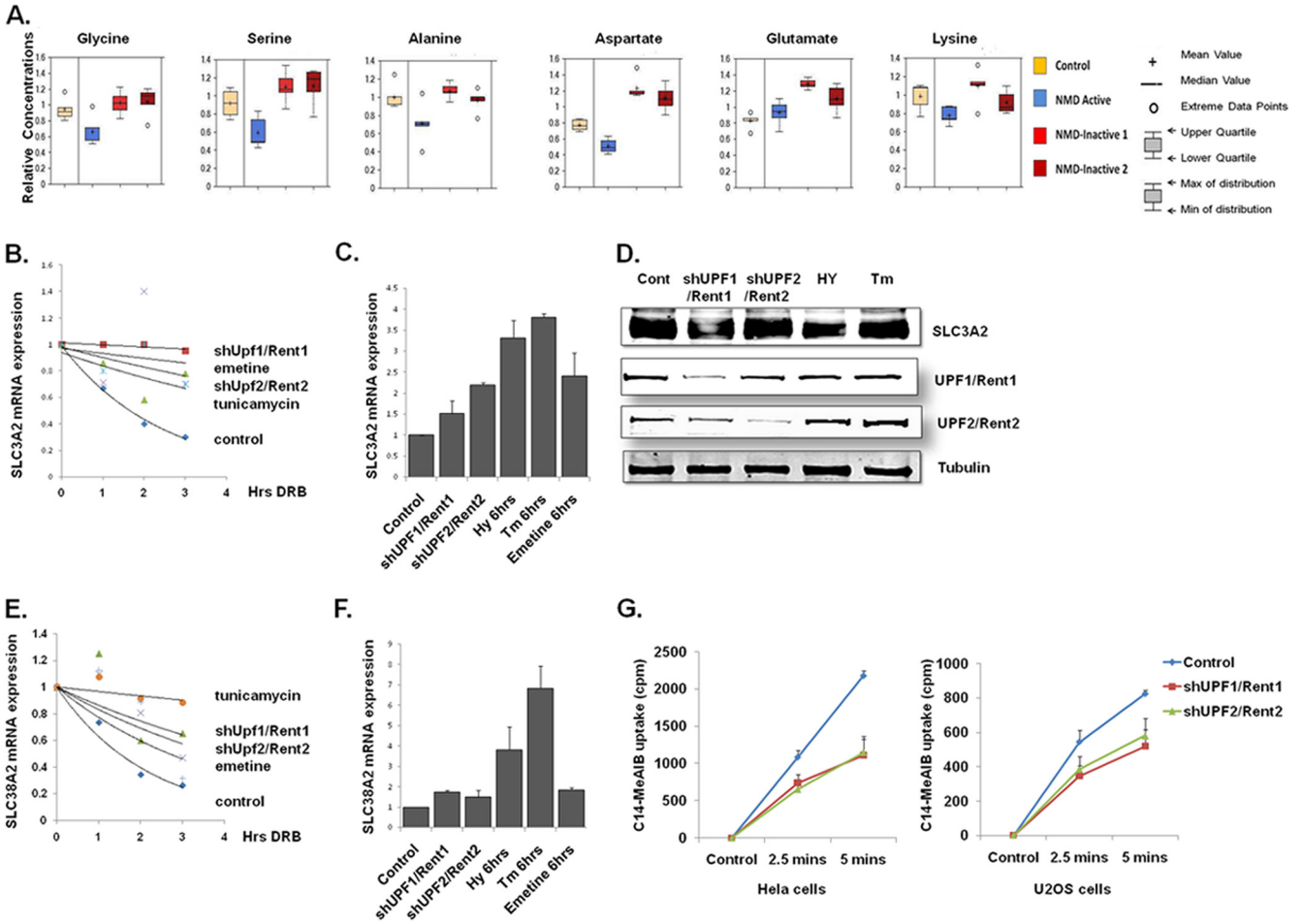

FIG 4 Inhibition of NMD augments intracellular amino acid concentrations. (A) Amino acids, assayed as discussed in the text, were determined in control U2OS cells, U2OS cells overexpressing Upf1/Rent1 (NMD active [NMD-A]), cells with Upf1/Rent1 depletion (NMD inactive [NMD-I1]), and cells with Upf2/Rent2 depletion (NMD inactive [NMD-I2]). (B and C) The stability (B) and expression (C) of SLC3A2 mRNA was determined in U2OS cells with NMD inhibited by Upf1/Rent1 or Upf2/Rent2 depletion by rendering the cells hypoxic (hy) or by treatment with tunicamycin (Tm) or emetine. (D) SLC3A2 protein expression was assessed in the cells described for panel B. (E and F) SLC38A2 mRNA stability (E) and expression (F) were assessed as described above. (G) MeIAB uptake into control, Upf1/Rent1-depleted, and Upf2/Rent2-depleted HeLa cells (left panel) and U2OS cells (right panel) was determined as described in the text.

tent with the model that many of the increased intracellular amino acids noted with NMD inhibition are primarily due to increased autophagy.

The combination of NMD inhibition and autophagy leads to cell death. We reasoned that if the induction of autophagy with NMD inhibition is an adaptive mechanism to rid the cell of misfolded, mutated, aggregated, and otherwise deleterious proteins, cells would not tolerate NMD inhibition in the absence of autophagy. Chloroquine, a clinically available and relatively nontoxic antimalarial, is well documented to block autophagy and is currently undergoing testing in clinical trials for a variety of neoplasms. Some cancer cell lines, including the colon cancer cell line HCT116, are known to be sensitive to autophagy inhibition even under nonstressed conditions, with increased apoptosis when autophagy is induced by cellular stress (13). We noted a modest decrease in cell viability (Fig. 5A) and increase in apoptosis (as noted by PARP cleavage) (Fig. 5B) when HCT116 cells were treated with chloroquine. There was a marked decrease in viability and a corresponding increase in apoptosis when HCT116 cells depleted of Upf1/Rent1 or Upf2/Rent2 were treated with chloroquine (Fig. 5A and B) compared to control HCT116 cells, demonstrating their inability to tolerate NMD inhibition when autophagy is repressed. In contrast, when NMD was hyperactivated by Upf1/Rent1 overexpression, cells were less sensitive to the effects of chloroquine (Fig. 5A).

We have recently identified a chemical inhibitor of NMD, ethyl-4,5-dimethyl-2-{[(3-oxo-1,2,3,4-tetrahydro-2-quinoxalinyl) acetyl]amino}-3-thiophenecarboxylate, designed to disrupt crucial interactions with components of the NMD complex (L. Martin and L. B. Gardner, unpublished data). This NMD inhibitor is minimally toxic in a variety of cell lines over 24 to 36 h (Martin et al., submitted). Over 48 h of treatment, the pharmacological inhibition of autophagy with chloroquine led to an 8% decrease in HCT16 viability, and the pharmacological inhibition of NMD led to 20% cell death. The combination of both inhibitors led to a 42% decrease in cell viability (Fig. 5C). These proof-of-concept experiments suggest that the simultaneous pharmacological inhibition of NMD and autophagy may be a viable therapeutic strategy to treat cancer.

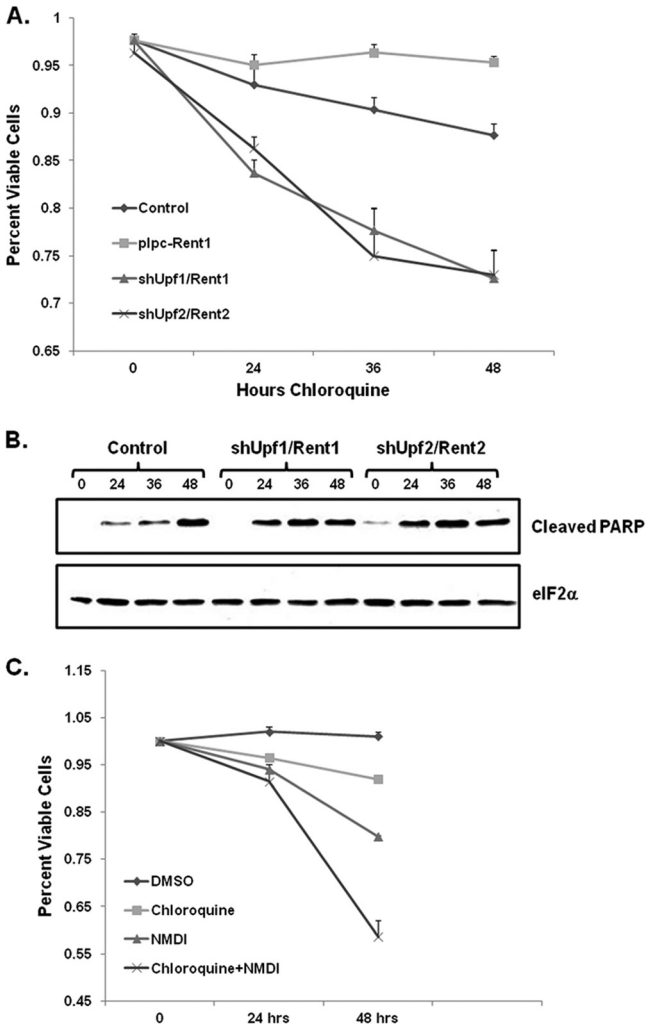

FIG 5 Viability with autophagy inhibition is decreased when NMD is also inhibited. (A and B) Control HCT116 cells and cells with hyperactivated NMD (Upf1/Rent1 overexpression) or inhibited NMD (Upf1/Rent1 or Upf2/Rent2 depleted) were treated with chloroquine for up to 48 h, and viability (A) and cleaved PARP (B) were assessed. (C) HCT116 cells were treated with chloroquine, 5 μM ethyl-4,5-dimethyl-2-{[(3-oxo-1,2,3,4-tetrahydro-2-quinoxalinyl)acetyl]amino}-3-thiophenecarboxylate, or both, and viability was assessed. DMSO, dimethyl sulfoxide.

DISCUSSION

Our data demonstrate that NMD activity regulates autophagy in a variety of primary and transformed cell lines. Specifically, the inhibition of NMD can induce autophagy, and the hyperactivation of NMD can blunt autophagy. The observation that autophagy is induced with NMD inhibition is consistent with data suggesting that the physiological inhibition of NMD not only is a response to stress but also contributes to the cellular adaptation to stress (3–5). We have previously reported that the stress-induced inhibition of NMD by eIF2α phosphorylation augments the ER stress/unfolded protein response pathway and improves cellular survival with ER stress (3, 5). Our data now suggest that ER stress not only activates an adaptive response by degrading proteins through ER-associated degradation (ERAD) but also leads to the degradation of cytoplasmic proteins through NMD inhibition and the induction of autophagy (23). Similarly, our data suggest that during the integrated stress response, eIF2α phosphorylation not only upregulates transcripts for amino acid transporters and genes involved in amino acid metabolism but also increases intracellular amino acids through NMD-induced autophagy (22, 24). While our current data rely primarily on cell lines and further *in vivo* studies are warranted, the dynamic regulation of NMD likely plays an important role in the *in vivo* activity of autophagy.

One important consequence of autophagy is the generation of free intracellular amino acids. We show that the suppression of NMD increases intracellular amino acids primarily through autophagy and not through the stabilization of ATF4 and a corresponding transcriptional increase of amino acid transporters or through the direct stabilization of amino acid transporter mRNAs. Although we demonstrate that many amino acid transporter mRNAs are direct and/or indirect targets of NMD, at least neutral amino acid transport is not upregulated by the inhibition of NMD, despite an increase in the mRNAs encoding the SNAT2 transporter. While we have not ruled out the possibility that other amino acids have their transport and/or metabolism directly regulated by NMD, our findings emphasize that the biological significance of an mRNA's degradation by NMD must be verified with evidence either that the protein is similarly regulated or that the stabilized mRNA has a biological function (as suggested for truncated antibody and T-cell receptors [14, 25]).

Our data are consistent with a model in which the cell activates autophagy to clear mutated, aggregated, and misfolded proteins which accumulate upon NMD inhibition. While it is difficult to quantitatively assess the expression of such proteins, multiple studies have demonstrated that alternatively spliced mRNAs accumulate with NMD inhibition (6–8, 10). Although the physiological/pathological inhibition of NMD occurs with eIF2α is phosphorylation, a condition that blunts translation, we have demonstrated that many mRNAs stabilized with NMD inhibition are still actively translated despite eIF2α phosphorylation, including functional proteins and maladaptive peptides (3, 5). Indeed, it is clear that the induction of autophagy with NMD inhibition is a protective response, as demonstrated by the observation that the simultaneous inhibition of NMD and autophagy triggers apoptosis. It is also possible that in addition to the stabilization of ATF4, aggregated proteins that accumulate with NMD inhibition contribute to the activation of autophagy, as has been suggested for the induction of autophagy in Huntington's disease, where the aggregation of polyglutamine (polyQ) length-dependent increases of cytoplasmic inclusions is able to induce autophagy in an mTOR-independent manner (26).

We have previously observed that NMD inhibition is necessary for transformed cells to grow in soft agar or as explants in mice (5). Gene expression studies suggest that many genes that play a role in tumor-related pathways are upregulated with NMD inhibition, and we have demonstrated that ATF4 protein and the unfolded protein response (both of which are required for tumor growth) are augmented by the physiological suppression of NMD (3–5, 24). Although the role that autophagy plays in tumorigenesis is complex, autophagy is thought to allow established tumors to adapt to the metabolic stresses of the tumor microenvironment (27). Since we have demonstrated that NMD is inhibited in three-dimensional tumors, NMD inhibition likely contributes to tumor autophagy (in addition to the direct activation of autophagy in tumors by nutrient deprivation through regulation of mTOR and other pathways) (5). Together, these findings support a model in

which the physiological inhibition of NMD by cellular stress contributes to three-dimensional tumor growth.

Despite the role of NMD inhibition in augmenting the cellular stress response, our present data suggest that when autophagy is inhibited, NMD inhibition is no longer protective but is detrimental. This model has potential therapeutic implications. While the constitutive deletion of genes necessary for NMD can result in embryonic lethality in mice, more modest suppression of NMD through gene depletion or the expression of dominant negative constructs, as well as the complete inhibition of NMD in differentiated cells, results in limited toxicities, suggesting that modest NMD inhibition may be well tolerated (5, 7, 8, 14, 17). The use of autophagy inhibitors has had modest efficacy in both preclinical and early clinical trials, likely because autophagy is both upregulated in tumors and necessary for tumor growth (28). For tumors in which NMD is not maximally inhibited, the administration of a pharmacological NMD inhibitor to induce autophagy with the coadministration of a pharmacological autophagy inhibitor could result in an selective and effective chemotherapeutic regimen.

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