Alcohol induces p53-mediated apoptosis in neural crest by stimulating the AMPK-mediated suppression of TORC1, p70/S6K, and ribosomal biogenesis.

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Abstract (236/250)

Background. Prenatal alcohol exposure is a leading cause of permanent neurodevelopmental disability. Diagnosis is often initiated by a distinctive craniofacial appearance that originates, in part, from the apoptotic deletion of craniofacial progenitors, a stem cell lineage called the neural crest (NC). We recently demonstrated that alcohol causes nucleolar stress in NC through its suppression of Ribosome Biogenesis (RBG) and this suppression is causative in their p53/MDM2-mediated apoptosis. Here, we show that this nucleolar stress originates from alcohol's activation of AMPK, which suppresses TORC1 and the p70/S6K-mediated stimulation of RBG. **Methods**. Alcohol-exposed cells of the pluripotent, primary cranial NC line O9-1 were evaluated with respect to their p70/S6K, TORC1, and AMPK activity. The functional impact of these signals with respect to RBG, p53, and apoptosis were assessed using gain-of-function constructs and small molecule mediators. **Results**. Alcohol rapidly (<2hr) increased pAMPK, p-Raptor, p-mTOR(S2446), and reduced both total and p-p70/S6K in NC cells. These changes persisted for at least 12hr to 18hr following alcohol exposure. Attenuation of these signals via gain- or loss-of-function approaches prevented alcohol's suppression of rRNA synthesis and the induction of p53-stimulated apoptosis. **Conclusions**. We conclude that alcohol induces ribosome dysbiogenesis and activates their p53/MDM2-mediated apoptosis via its activation of pAMPK, which in turn activates Raptor to suppress the TORC1/S6K-mediated promotion of ribosome biogenesis. This represents a novel mechanism underlying alcohol's neurotoxicity and is consistent with findings that TORC1/S6K networks are critical for cranial NC survival.

1. Introduction

Prenatal alcohol exposure (PAE) is a leading cause of permanent neurodevelopmental disability that features deficits in cognition and executive function [1]. Diagnosis is often initiated by a distinctive craniofacial appearance [4] that has complex origins reflecting both changes in the underlying brain growth [5,6] and direct effects upon the cranial neural crest (NC) [7,8], a pluripotent stem cell linage that forms the facial bone and cartilage, and neuronal elements including certain cranial nerves, Schwann cells, and the sympathetic and parasympathetic system [9]. NC induction begins at neurulation and, shortly thereafter, the NC emigrate from the closing dorsal neural tube and into peripheral tissues where they differentiate into the aforesaid structures. Pharmacologically relevant alcohol exposure (10-80mM) disrupts multiple events in cranial NC development including their induction, migration, proliferation, and survival [6-8]. We and many others have shown that alcohol causes the apoptotic deletion of NC progenitors at a specific phase of their development, at the onset of migration [10-14]. Alcohol interacts with multiple pathways to modulate facial outcome and/or NC death including sonic hedgehog, retinoid, oxidative stress, Wnt, and p53, among others [7,8,16-24]. However, the regulatory mechanisms governing NC sensitivity to alcohol remain elusive. Novel insight into this apoptotic mechanism emerged from our whole exome sequencing of NC progenitors, wherein a brief alcohol exposure (2hr, 52mM) rapidly (<6hr) repressed the expression (padj 10E-47) of 106 nuclear and mitochondrial ribosomal proteins (RP) by 30%-50% [31]. A similar repression of RPs is observed in alcohol-exposed zebrafish [32], headfolds of mouse, [33,34] and chick strains [35] having differential alcohol vulnerability, primary mouse neural stem cells [36] and in the NC primary cell line O9-1 [37]. Thus, suppression of RPs is a consistent feature of alcohol-exposed NCs and neuroprogenitors across taxons.

Why would a suppression of RPs lead to the apoptosis of cranial NC populations? For rapidly proliferating populations such as NC, each cell division requires the duplication not only of DNA, but the ribosomes that accomplish protein synthesis. Indeed, the production of rRNA plus the ~200 proteins and short RNAs involved in rRNA processing and assembly into ribosomes is estimated to occupy 70-80% of the cellular energy budget, and is seen visually in the nucleosomes, which are the multiple operon sites of this ribosome biogenesis (RBG) [38,39,40]. Unsurprisingly, cells have coopted RBG to monitor their internal stress, and RBG is tightly coupled to cellular anabolism and the p53 checkpoint pathway [41-43]. Specifically, under normal conditions RBG is stimulated by the anabolic effector TORC1 via p70/S6K [41-43]. When energy is limiting, pAMPK phosphorylates the TORC1 components RAPTOR and TSC1/2 to suppress TORC1 and RBG, thus integrating RBG with cell metabolism. Under stressors that reduce the metabolites necessary for rRNA synthesis, RBG ceases and the nucleoli visually disappear [44-48]. This process, known as "nucleolar stress", is linked to p53 via nuclear E3 ubiquitinase MDM2, which under normal conditions targets and destabilizes p53 [49-54]. Under nucleolar stress, reduced rRNA synthesis enables a RPS5-RPL11-5SrRNA complex to bind and inhibit MDM2, thus allowing p53 to become transcriptionally active [49-54]. The importance of RBG for NC is evidenced in the genetic disorders known as ribosomopathies, in which loss-of-function in either AMPK – TORC1 – p70/S6K signaling, or in components of RBG, causes p53-mediated NC losses and facial deficits that share similarities with those of PAE (i.e. Treacher-Collins, Diamond-Blackfan Anemia) [55-61].

We recently reported that alcohol exposure impairs ribosomal biogenesis (RBG) to induce nucleolar stress in early cranial NC progenitors. This includes the rapid cessation of rRNA synthesis and dissolution of nucleolar structures in response to pharmacologically relevant

alcohol exposures (20-80mM for 2hr) [37]. This was followed by the stabilization of nuclear p53 and their subsequent p53-mediated apoptosis. Haploinsufficiency of RPL5, RPL11, or RPS3A using a morpholino approach synergized with alcohol to cause craniofacial deficits in a zebrafish model of PAE [37, Berres], and transfection with MDM2 or loss-of-function p53 blocked these effects of alcohol. However, the mechanism responsible for alcohol's suppression of RBG is unknown. Here we test the hypothesis that alcohol represses RBG through its suppression of p70/S6K via TORC1, and that its activation of AMPK mediates both the loss of TORC1/p70/S6K signaling and their p53-mediated apoptosis.

2. Materials and Methods

2.1. Cell Culture

All studies use the established primary cranial NC line O9-1 (#SCC049, Millipore; Burlington, MA) [62,90]. This non-transformed stem cell line was originally isolated from mass culture of primary cranial NCs isolated at embryonic day (E)8.5 from C57BL/6J mouse embryos that expressed Wnt1-Cre:R26R-GFP [62]. It expresses key NC markers (e.g., Twist1, Snail1, Nestin, CD44), can differentiate into osteoblasts, chondrocytes, smooth muscle cells, tooth pulp, and glial cells, but not neuronal cells, and develop normally when added back to embryos [62,87,91,92]. We find it has alcohol responses identical to those of primary avian or mouse NCs [37]. Cells were maintained on Matrigel-coated plates in their pluripotent state at 50-60% confluence in DMEM (Gibco, Grand Island NY) supplemented with 6% ES cell media (ES-101B, Sigma, Louis MO), 15% fetal calf serum (Millipore), 0.1mM nonessential amino acids (Gibco), 1000 U/ml leukemia inhibitory factor (LIF, Gibco), 25ng/ml basic fibroblast growth factor (Invitrogen, Waltham, MA), 0.3μM β-mercaptoethanol (MP Biomedical, Solon, OH), and

100 U/ml penicillin and streptomycin (Gibco). Experiments were performed in the identical media but containing 1% FCS conditioned media and used for less than 24hr.

2.2. Alcohol Exposure

Cells were treated with USP grade ethanol (Koptec, King of Prussia, PA) at concentrations ranging from 20 to 80mM. We find that the alcohol concentrations are less than 10mM by 2hr post-addition. Thus, these studies model acute binge exposure.

2.3. Quantitative PCR

RNA was isolated using Trizol reagent (Invitrogen) at experimentally determined times following exposure to 80mM alcohol. 1µg of total RNA was reverse transcribed with 500µg/ml random primer (#C1181), 5X ImProm II reaction buffer (#M289A), ImProm II (#M314A), 25 mM $MgCl_2$ (#A351H), 10 mM dNTP (#U1511), 20U RNAsin (#N2511; all from Promega, Madison, WI) and RNAse free water. Quantitative PCR (qPCR) were performed using SYBR Select Master Mix (ABI, #4472913) and the Real-Time PCR system (Bio-Rad CFX96; Hercules, CA). *De novo* rRNA synthesis was quantified by qPCR for the Internal Transcribed Spacer ITS1 (138bp) located between 18S and 5.8S rRNA. [36,37] Primers (IDT, Coralville, IA) for the internal transcribed spacer 1 (ITS-1) of 47S pre-rRNA (forward: 5′-CCGGCTTGCCCGATTT-3′, reverse: 5′-GCCAGCAGGAACGAAACG-3′) and β2 microglobulin (forward: 5′-TTCACCCCCACTGAGACTGAT-3′; reverse: 5′-GTCTTGGGCTCGGCCATA-3′) were used. Relative abundance was calculated using the $2^{-\Delta\Delta CT}$ method and was normalized to β2 microglobulin.

2.4. Western Immunoblot Analysis

Total proteins were isolated from cells using NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO3, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride; all from Sigma). Total protein was separated on 10% SDS-PAGE reducing gels or 4%-20% detergent gels (BioRad). The resolved proteins were transferred onto a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). The primary antibodies were directed against p-AMPK Thr-172 (#2535), AMPK (#2793), p-mTOR Ser2446/2448 (#2971), mTOR (#4517), p-Raptor Ser 792 (#2083), Raptor (#2280), p-s6k1 Thr-389 (#9234), s6k1(#9202), p-4EBP1 Thr70 (#9455), 4EBP1(#9452; all used at 1:1000 from Cell Signaling; Danvers MA), P53 (#ab26, 1:500, Abcam; Cambridge, England, UK), and GAPDH (#G8795, 1:2500; Sigma). The secondary antibodies were goat anti-rabbit (#4010-05, 1:5000) and anti-mouse (#1030-05, 1:5000, both from Southern Biotech, Birmingham AL) coupled to horseradish peroxidase. Signal was imaged by chemiluminescence using the Radiance Q detection system (Azure Biosystems, Dublin, CA).

2.5. Cell Transfection

Cells were transiently transfected at 80% confluence with plasmid pcDNA3 p70/S6K2 E388 D3E (#17731; Addgene; Watertown, MA; Lee-Fruman 1999) and Lipo-3000 (#L3000001; Invitrogen) according to the manufacturer's instructions. This construct contains four gain-of-function mutations (T388E, S410D, S417D, S423D) to make it rapamycin-resistant and constitutively active (ca-S6K; Lee-Fruman 1999). Cells recovered for 6hr prior to starting the alcohol exposure.

2.6. Apoptosis Analysis

Cells were treated with 80 mM alcohol. At 16hr thereafter they were fixed in 4% paraformaldehyde, permeabilized in 0.2% TritonX-100, and apoptosis was detected using in situ cell death detection kit (TMR Red; Roche, Indianapolis, IN). Nuclei were visualized using DAPI. Images were taken under uniform exposure. Data are the means of three independent experiments, each having three samples per group and quantifying three images per sample.

2.7. Small Molecule Interventions

These used the AMPK inhibitor dorsomorphin (5μM), the AMPK activator AICAR (500μM) or the TORC1 inhibitor rapamycin (1 nM; all from Selleck Chemicals, Houston TX). Cells were exposed for 1h and then treated with 80mM alcohol and collected at experimentally determined times thereafter.

2.8. Statistical Analysis

Data were tested for normality and then analyzed using the appropriate test, Student's t-test for 2 groups comparisons, and the ONE-WAY ANOVA test for multiple comparisons. $P < 0.05$ was considered significant. Results are presented as mean ± SEM of 3 samples per group.

3. Results

3.1. Alcohol suppresses RBG and induces nucleolar stress and p53-mediated apoptosis in NC.

A time course reveals that exposure to 80mM alcohol suppresses de novo rRNA synthesis by 1hr following exposure (CON 100 ± 11.7%, ALC 63.5 ± 4.8%; $p<0.001$; **Figure 1A**). This reduction

persists for at least 4hr to 8hr post-exposure. This is accompanied by the dissolution of nucleolar structures, as visualized using antibodies directed against the RNA polymerase I transcription factor UBF (**Figure 1B**). By 2hr, p53 protein is stabilized in NC and its content increases 70 ± 11%, and it remains elevated for at least 12hr (CON 100 ± 42%, ALC 191 ± 14%; $p<0.001$; **Figure 1C**). By 16hr post-exposure, the alcohol-exposed NC undergo apoptosis (CON 0.73 ± 0.35%, ALC 10.94 ± 1.72%; $p<0.001$; **Figure 1D**), and we showed previously [37] that this apoptosis is p53-dependent.

3.2. Alcohol exposure reduces p70/S6K activity and S6K gain-of-function normalizes rRNA synthesis and apoptosis in NC.

Alcohol exposure reduced the abundance of p70/S6K in a complex manner. With respect to total p70/S6K protein, its content was 69 ± 6% within 2hr of alcohol exposure, and these reductions persisted through 6hr and were normalized by 18hr post-exposure (**Figure 2A**). This was accompanied by a reduction in p70/S6K expression to 89.7% of controls (padj=4.28E-12) as determined by whole exome sequencing. With respect to the activated form, phospho-p70/S6K-Threonine(T)389, its levels did not decline as a percentage of total p70/S6K until 12hr post-exposure, when levels were at 57 ± 1% at 12hr and 59 ± 7% at 18hr post-exposure. However, because of the absolute decline in p70/S6K, this represents an absolute decline in the abundance of activated p70/S6K within 2hr of the alcohol exposure.

To test the functional involvement of the p70/S6K loss, we transfected NC with a constitutively active (ca) version of p70/S6K, which contains four gain-of-function mutations to make it rapamycin-resistant (Lee-Fruman 1999). As expected, the ca-p70/S6K construct countered the alcohol-mediated reduction in p70/S6K(T389) (ALC, 55 ± 7%, ALC+caS6K, 83 ±

12%, p=0.022; **Figure 2B**). It restored de novo rRNA synthesis in alcohol-exposed cells (ALC 49 ± 5%, ALC+caS6K 90 ± 3%, p<0.001; **Figure 2C**); unexpectedly, it reduced rRNA synthesis in control cells (CON, 100 ± 6%, CON+caS6K, 54 ± 1%, p<0.001). It also abrogated alcohol's activation of p53 (ALC, 155 ± 29%, ALC+caS6K, 106 ± 14%; p=0.05; **Figure 2D**) and its induction of their apoptosis (**Figure 2E,** ALC, 2.70 ± 0.45%, ALC+caS6K, 0.52± 0.11%; p <0.001). We conclude that alcohol's reduction in p70/S6K and p70/S6K are consistent with its inhibition of RBG.

3.3. Alcohol activates pRAPTOR to repress TORC1 signaling activity.

A primary activator of p70/S6K is the Target of Rapamycin Complex 1 (TORC1). TORC1 is under complex regulation via phosphorylation of its component mTOR by kinases including Akt, AMPK, and p70/S6K itself. Under alcohol exposure, we found increased phosphorylation of mTOR at the dual sites S2446/S2448 (**Figure 3A**); this increase was observed within 2hr of alcohol exposure and persisted for at least 12hr (**Figure 3B**). Although these two sites could not be distinguished, they are under quite different regulation; S2446 is targeted by AMPK and S2448 by both Akt and p70/S6K (ref); increased targeting by p70/S6K was inconsistent with data indicating that alcohol reduced its activity. To further explore alcohol's impact on them we evaluated Raptor, which is an AMPK-dependent negative regulator of TORC1. This revealed an increase in p-Raptor(S792) by 6hr of exposure (CON, 100 ± 5%, ALC. 168 ± 29%; p=0.04; **Figure 3C**) and it remained elevated for 12hr to 18hr (ALC 12hr, 143 ± 2%, p<0.001 vs CON; ALC 18hr, 141 ± 25%, p<0.10 vs. CON; **Figure 3D**). Consistent with an increase in this negative regulator of TORC1 was a reduction in p4E-BP1 (CON, 111 ± 6%, ALC 85 ± 4%, p = 0.027), which is otherwise activated by TORC1 to increase protein synthesis in parallel with p70/S6K's

actions on RBG. We also explored alcohol's impact on other elements of TORC1 and TORC2 and found no differences with respect to the abundance of pRictor, Deptor, pTSC2, or pPKCα (**Figure 3E**).

To complement this work, we tested if inhibition of the TORC1 signaling pathway was sufficient to induce NC apoptosis. Pretreatment of alcohol-exposed cells with rapamycin (Rapa), a small molecule inhibitor of TORC1 [22], further suppressed rRNA synthesis (ALC, 80 ± 0.3%, ALC+Rapa, 59 ± 13%; p=0.03; **Figure 3F**), and aggravated p53 stabilization (ALC, 151 ± 21%, ALC+Rapa, 175 ± 8%; p=0.05; **Figure 3G**) and apoptosis (ALC, 5.19 ±0.37%, ALC+Rapa, 7.48± 0.76%; p=0.004; **Figure 3H**). Interestingly, rapamycin increased the p53 content in control cells but did not suppress rRNA synthesis or cause apoptosis in control cells, indicating that TORC1 inhibition was not sufficient, in of itself, to suppress RBG and induce their apoptosis. Taken together, these data suggested that acute alcohol rapidly repressed TORC1 activity and this might be mediated thru the AMPK-mediated activation of Raptor.

3.4. Alcohol rapidly activates AMPK signaling in NC, and its inhibition prevents alcohol's suppression of RBG and p53-mediated apoptosis.

Consistent with the reductions in TORC1 and p70/S6K, alcohol caused a rapid rise in the activated form of AMPK, phosphor-AMPK(Thr-172), within 2hr of exposure (CON, 100 ± 1%, ALC 143 ± 6%, p<0.001; **Figure 4A,B**). Their pAMPK content peaked between 6hr and 12hr (6hr, 188 ± 10%; 12hr, 184 ± 15%; p<0.001) and remained elevated through at least 18hr (150 ± 14%, p<0.001) following the alcohol exposure. To functionally test AMPK's role in the loss of p70/S6K and RBG, and this p53-mediated apoptosis, we pretreated cells with the AMPK inhibitor dorsomorphin prior to their alcohol exposure. Dorsomorphin attenuated alcohol's

activation of pAMPK (ALC, 128 ± 7%, ALC+DOR, 89 ± 11%, p=0.005) and normalized their content of p-p70/S6K (ALC, 67 ± 6%, ALC+DOR, 135 ± 18%, p = 0.007), p-Raptor (ALC, 122 ± 8%, ALC±DOR, 27 ± 3, p<0.001), and p-mTOR (ALC 135 ± 6%, ALC+DOR, 69 ± 3%, p<0.001; **Figure 4C**). The AMPK inhibitor also normalized de novo rRNA synthesis (ALC, 70 ± 4%, ALC±DOR, 97 ± 6%, p<0.001; **Figure 4D**), prevented the alcohol-associated stabilization of p53 (ALC, 128 ± 5%, ALC±DOR, 58 ± 8%; p=0.003; **Figure 4E**), and prevented their apoptosis in response to alcohol (ALC 6.74 ± 0.92%, ALC+DOR 0.86 ± 0.06%; p <0.001; **Figure 4F**).

We then asked if inappropriate activation of pAMPK was sufficient to invoke their nucleolar stress and p53-mediated apoptosis. Using the AMP mimetic and AMPK activator AICAR, we found that it non-significantly elevated pAMPK (CON, 100 ± 6%, CON+AICAR, 127 ± 13%, p = 0.094; **Figure 5A**) and reduced p-p70/S6K (CON, 100 ± 26%, CON+AICAR, 64 ± 31%, p >0.1; **Figure 5B**) in otherwise normal cells, and it further elevated pAMPK (ALC, 133 ± 16%, ALC+AICAR, 172 ± 27%, p = 0.001) and trended to further reduce p-p70/S6K content in those exposed to alcohol (ALC, 38 ± 16%, ALC+AICAR, 24 ± 4%, p = 0.06). However, it was not sufficient to suppress rRNA synthesis in normal cells, nor did it stabilize p53 or cause their apoptosis (**Figure 5C-E**). In alcohol-exposed cells, it did not further suppress rRNA synthesis, it modestly increased p53 (ALC, 120 ± 4%, ALC+AICAR, 140 ± 9%, p = 0.03), and synergized with alcohol to worsen their apoptosis (ALC, 5.24 ± 0.50%, ALC+AICAR, 10.15 ± 0.51%, p <0.001). Taken together, these data indicate that alcohol's activation of AMPK is responsible for the p70/S6K-mediated loss of RBG and activation of p53-mediated apoptosis in NC.

4. Discussion

The most important finding reported here is that alcohol exposure suppresses the TORC1-dependent activity of p70/S6K in cranial neural crest to inhibit their ribosome biogenesis, induce nucleolar stress, and effect their p53-mediated apoptosis. Moreover, this suppression of p70/S6K was driven by alcohol's activation of pAMPK. Administration of pS6K gain-of-function or dorsomorphin-mediated inhibition of pAMPK were sufficient to sustain RBG in alcohol's presence and thus prevent p53 stabilization and apoptosis. Alcohol's action on this pathway was rapid, with a loss of rRNA synthesis by 1hr, and increases in both pAMPK and p53 within 2hr post-exposure. We reported previously that pharmacologically relevant alcohol exposures (20-80mM) suppress RBG and induce nucleolar stress in CNCs, leading to their p53/MDM2 mediated apoptosis. The rapidity of these responses by pAMPK, and their blockade or mirroring by AMPK and p70/S6K loss- and gain-of-function interventions, suggests that AMPK activation and its downstream suppression of TORC1/S6K are primary effectors of this alcohol-induced apoptosis.

A potential involvement of TORC1 in alcohol-mediated neural crest death has been suggested from prior studies, but those observations have not yet been integrated into the larger, cohesive mechanism investigated here. Specifically, in a zebrafish model of PAE, haploinsufficiency in Pdgfr synergized with alcohol to cause craniofacial deficits, and these were attenuated by treating the embryos with the TORC1 activator L-leucine (McCarthy 2013), as it can operate downstream of PDGFR. Because the study did not directly access TORC1 activity, alcohol's potential impact in that model is unclear. Acute alcohol exposures reduce TORC1 signaling in adult cerebral cortex and cerebellum (Wang 2018), similar to that here. Using the same antibody reagent as used here (#2971, Cell Signaling), chronic exposure reduced p-mTOR

in fetal hippocampus (Lee 2020); however, this was accompanied by elevated p-p70/S6K and p-4E-BP1, along with elevated Deptor and Rictor but not Raptor, outcomes distinct from those here. It is possible that chronic alcohol exposure led to adaptive or compensatory changes in response to reduced p-mTOR, or dysregulated aspects of that pathway (Lee 2020). Our findings are consistent, however, with the increased ULK/ATG signals in alcohol-exposed cells (Luo 2014; Chen Luo 2012), as autophagy also increases under TORC1 suppression; this has been posited to reflect a protective response to alcohol (Luo 2014; Chen Luo 2012). Acute alcohol (18-24hr) also suppresses phosphorylation of p70/S6K and 4E-BP1 in SH-SY5Y neuroblastoma cells [25] and C2C12 myocytes [18] and promoted apoptosis in the neuronal model [25]. The critical importance of TORC1 signaling to neural crest is highlighted by studies in which its modulation by genetic [15] or dietary means [24] disrupted craniofacial morphogenesis and altered the facial appearance. Taken together, the critical importance of RBG in sustaining the high proliferative rate of premigratory cranial NC [Warner 2001; Burstyn-Cohen 2002; Ridenour 2014], and the requirement for TORC1 to support p70/S6K activation and RBG [Chauvin 2014], would explain how the loss of TORC1 and p70/S6K by alcohol would enhance their vulnerability to alcohol's neurotoxicity.

As an anabolic effector of cell growth, TORC1 activity is tightly regulated by both its own direct nutrient sensing and by upstream kinases such as AMPK that further act to integrate metabolic status. As a primary suppressor of TORC1 and RBG [29, 41], alcohol's activation of pAMPK and its target pRAPTOR indicates that alcohol reduced RBG via repression of TORC1 signaling. This was further endorsed in that treatment of ALC NC with the AMPK antagonist dorsomorphin reversed alcohol's impact to normalize pAMPK, pS6K, p-mTOR, and pRAPTOR, and prevented both nucleolar stress and apoptosis. That AMPK inhibition blocked alcohol's

adverse effects suggest that pAMPK is necessary for their apoptosis. However, that neither rapamycin nor AICAR suppressed RBG or caused apoptosis in otherwise healthy NC indicates that signals in addition to pAMPK contribute to their nucleolar stress and apoptosis. This is not surprising as alcohol does not act through a single receptor, but rather binds and modulates the activity of multiple protein targets. For example, we have previously shown that in NC alcohol initiates a G-protein-mediated intracellular calcium transient that activates CaMKII and destabilizes nuclear β-catenin to trigger their apoptosis [25-27]. Alcohol also has been shown to modulate facial outcome through signals involving sonic hedgehog, retinoids, and oxidative stress, among others [7,8,16-24]. Studies are underway to elucidate these additional contributors that further destabilize these NC and tip them into an apoptotic fate.

Alcohol has been shown to increase pAMPK in multiple models including neurons of the hippocampus [11] and prefrontal cortex [30], isolated cardiac and myoblast cells [21,31,32], hepatocytes [20], and blockade of AMPK activation prevents the death of neuronal cells in models of zinc-induced toxicity [33] and Parkinson's disease [34]. However, how alcohol increases AMPK is currently unclear. AMPK is a master sensor and coordinator of cellular energy status and is activated in response to falling energy status via multiple pathways [69-72] including binding to elevated AMP, carbohydrate binding motifs [71,72] and, of relevance to our model, pCAMKK2 in response to intracellular calcium signals [73]. There is debate whether its activation in response to alcohol represents a beneficial or deleterious effect [9], and studies are contradictory in this regard. AMPK can increase glycolytic flux in response to hypoxia, as well as acting to limit energy-demanding processes such as RBG. This latter may be especially detrimental to cranial neural crest given their absolute dependence upon RBG, evidenced in the genetic disorders known as ribosomopathies, in which RBG loss-of-function causes p53-

mediated NC losses and facial deficits that parallel those of PAE (i.e. Treacher-Collins, Diamond-Blackfan Anemia) [55-61]. What is unclear is why alcohol's suppression of RBG invokes a nucleolar stress instead of a 'soft landing' or adaptive response that protects against p53 activation and MDM2/p53-mediated apoptosis, and investigations into this are underway.

In conclusion, pharmacologically relevant alcohol exposures significantly enhance the phosphorylation of AMPK in cranial NC and inhibit the downstream TORC1 signaling pathway to reduce p-p70/S6K and p-4E-BP1. This is accompanied by a suppression of de novo rRNA synthesis, induction of nucleolar stress, and stabilization of p53 to initiate their apoptosis. These findings underscore the pivotal role of the AMPK/TORC1 signaling axis as a novel mechanism in mediating alcohol's deleterious effects on neural crest development and survival.

5.0. Acknowledgements

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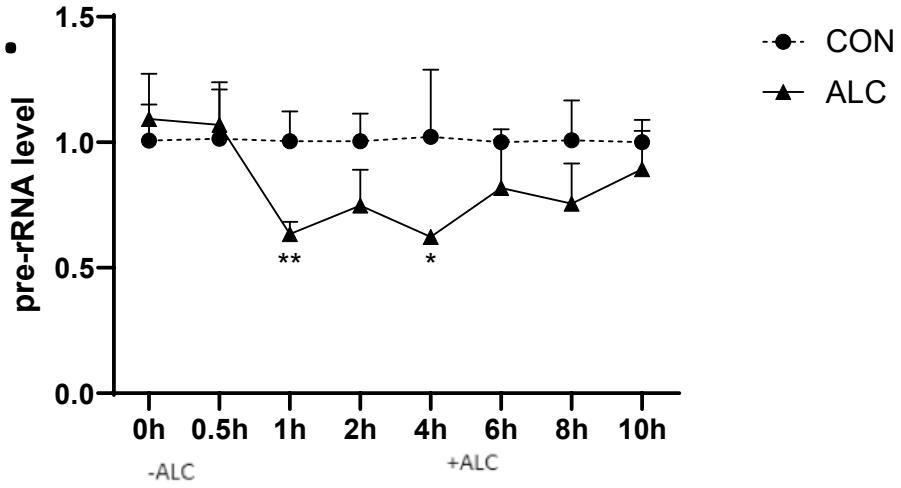
Alcohol induces p53-mediated apoptosis in neural crest by stimulating the AMPK-mediated suppression of TORC1, p70/S6K, and ribosomal biogenesis.

Yanping Huang, George R. Flentke, Susan M. Smith

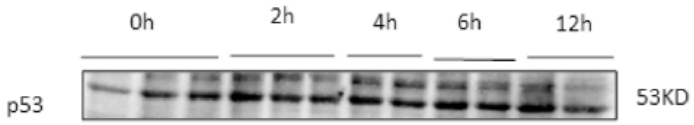
Figures 1-5

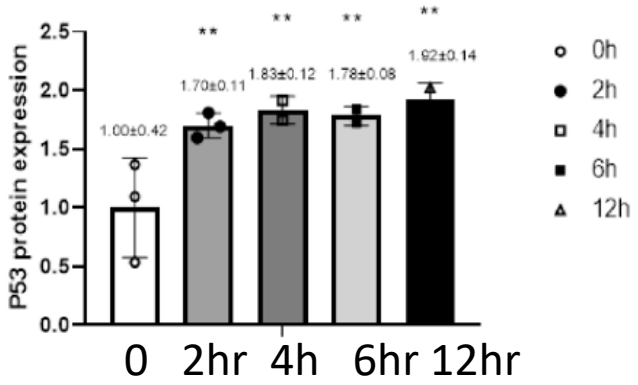
Figure 1. Alcohol represses ribosome biogenesis and causes p53-mediated apoptosis in primary neural crest.

A.



C.





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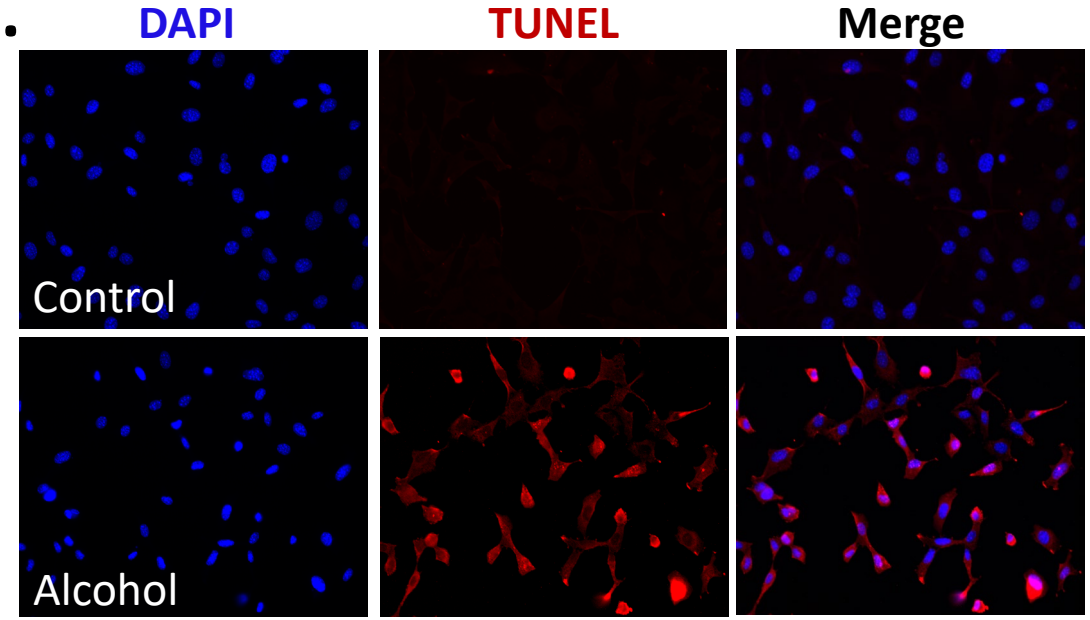


B. Insert nucleolar stress images

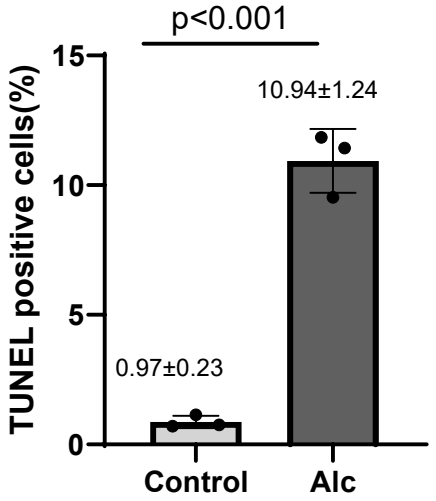


Figure 2A. Alcohol exposure reduces the RBG effector p70/S6K1 in NC.

A.

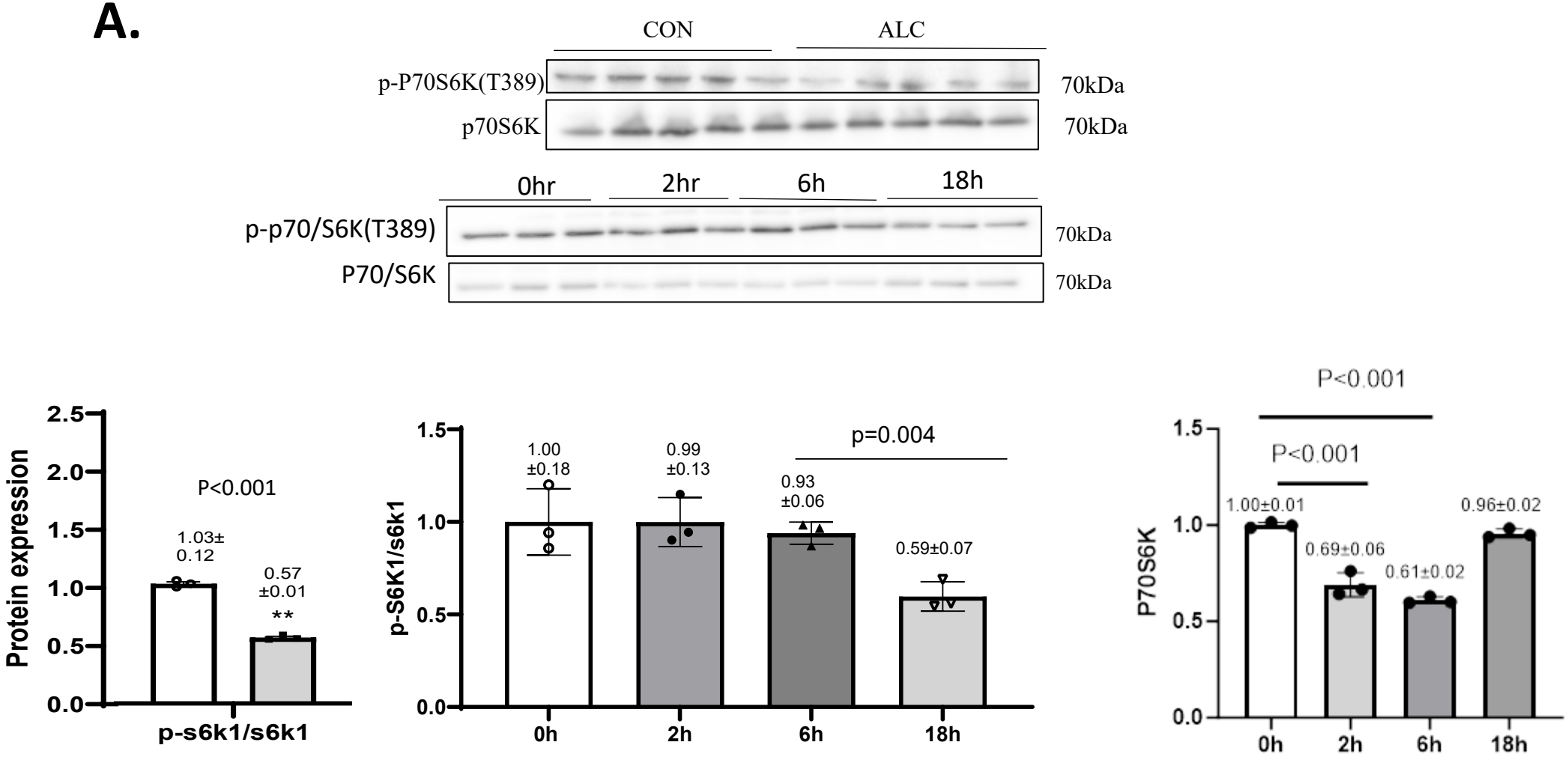
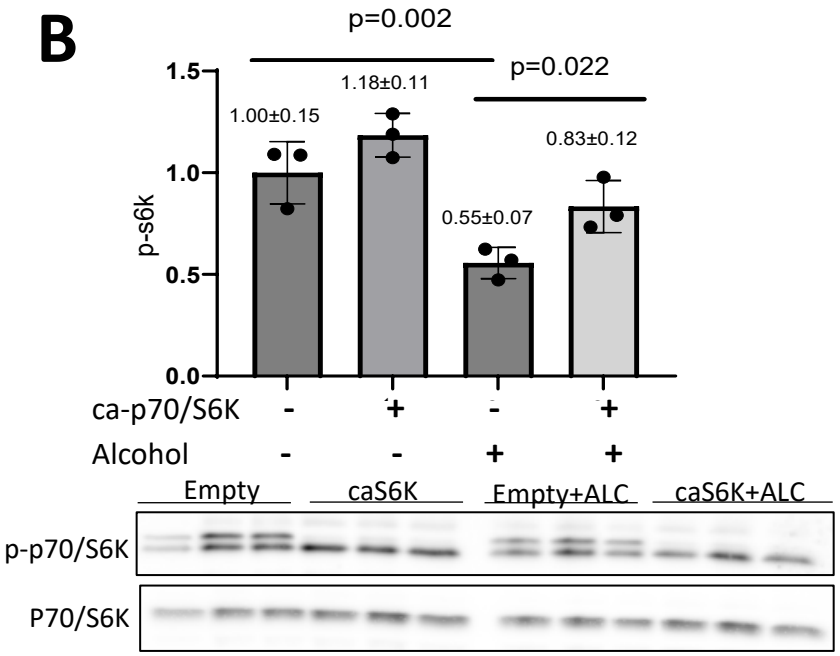
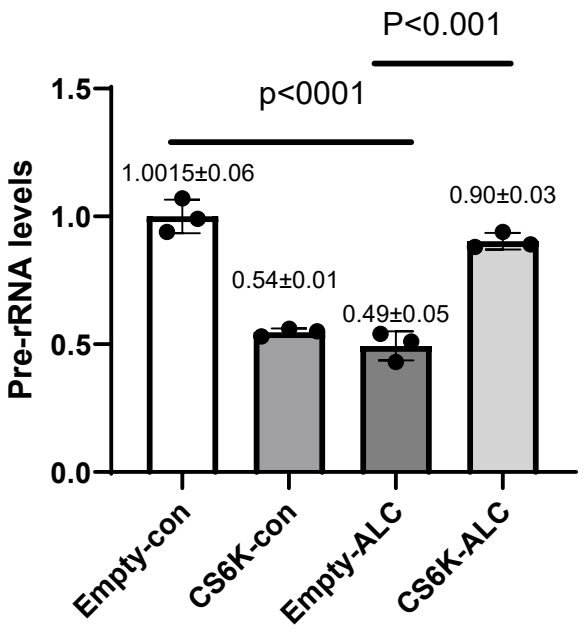
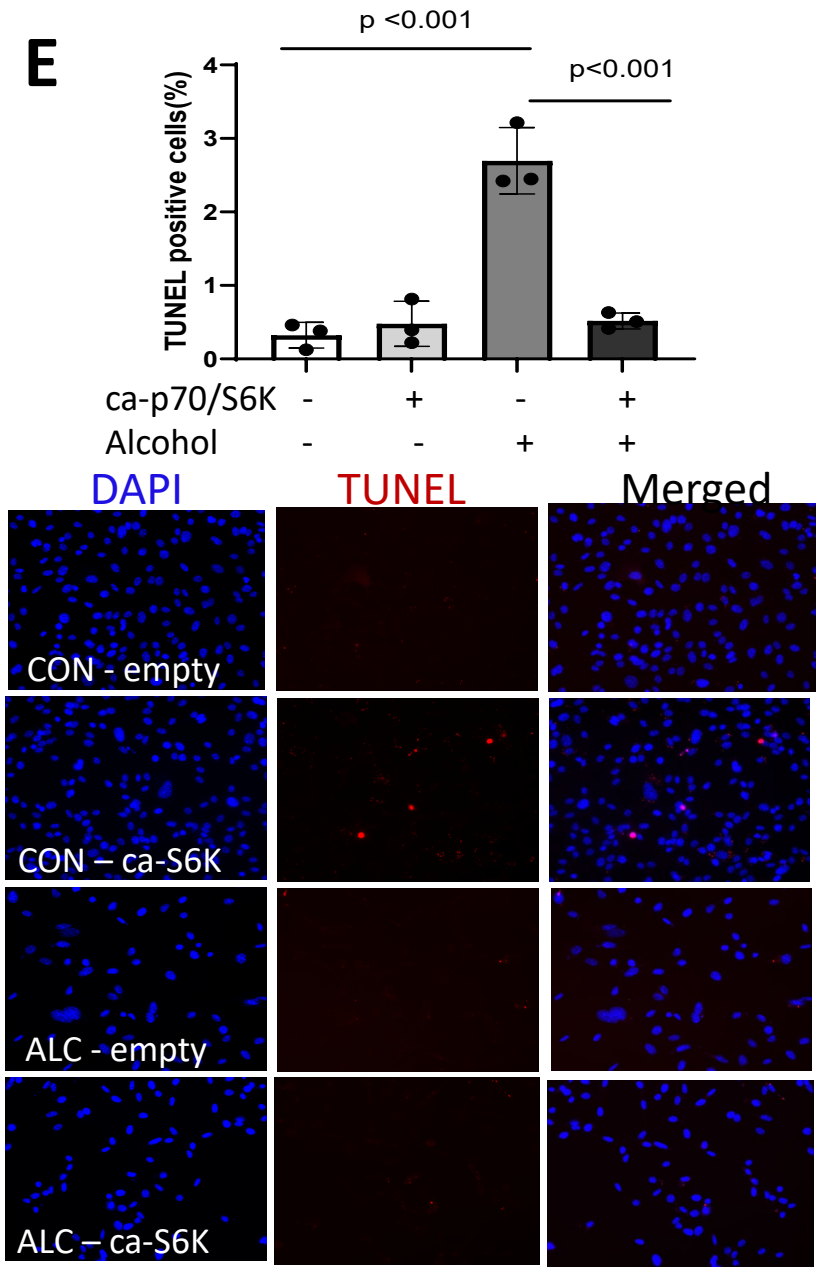


Figure 2B-E. Gain-of-function p70/S6K normalizes S6K & p53, and prevents the loss of rRNA synthesis and apoptosis in alcohol-exposed NC.







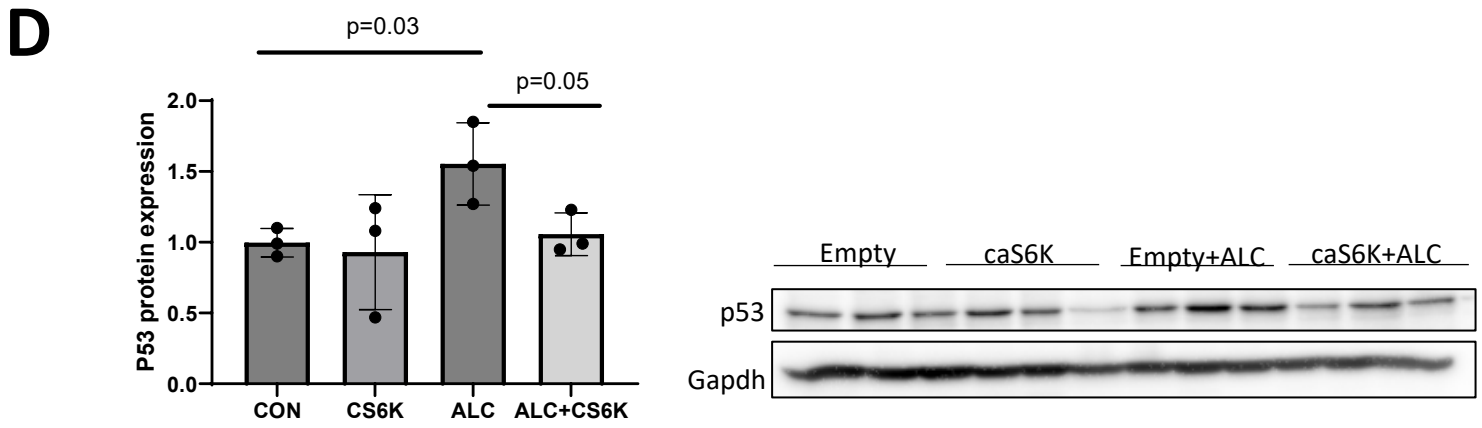
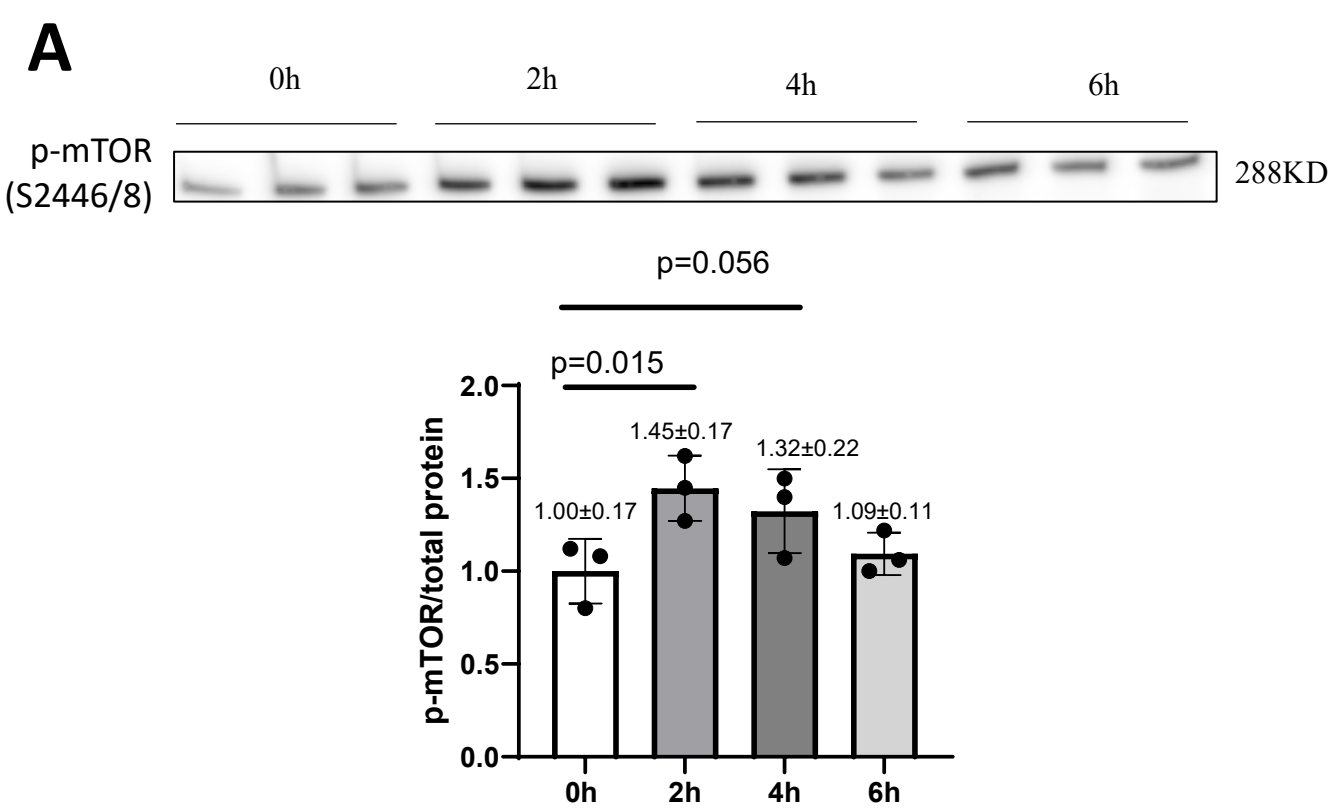


Fig. 3A,B. Phosphorylation of mTOR, an effector of p70/S6K, is increased at Ser2446/2448.



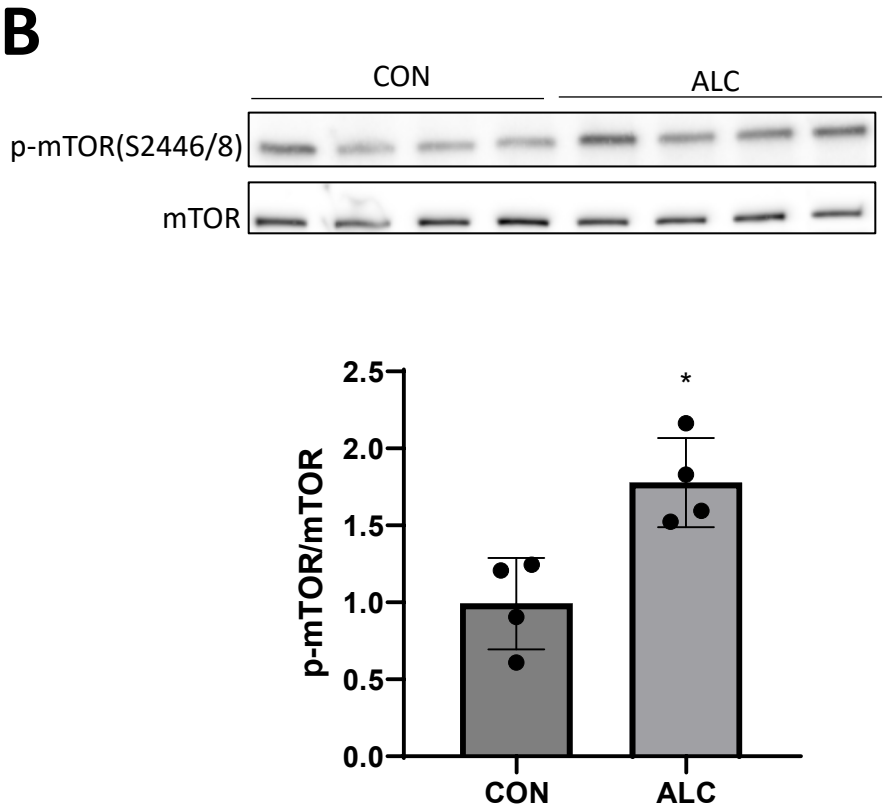
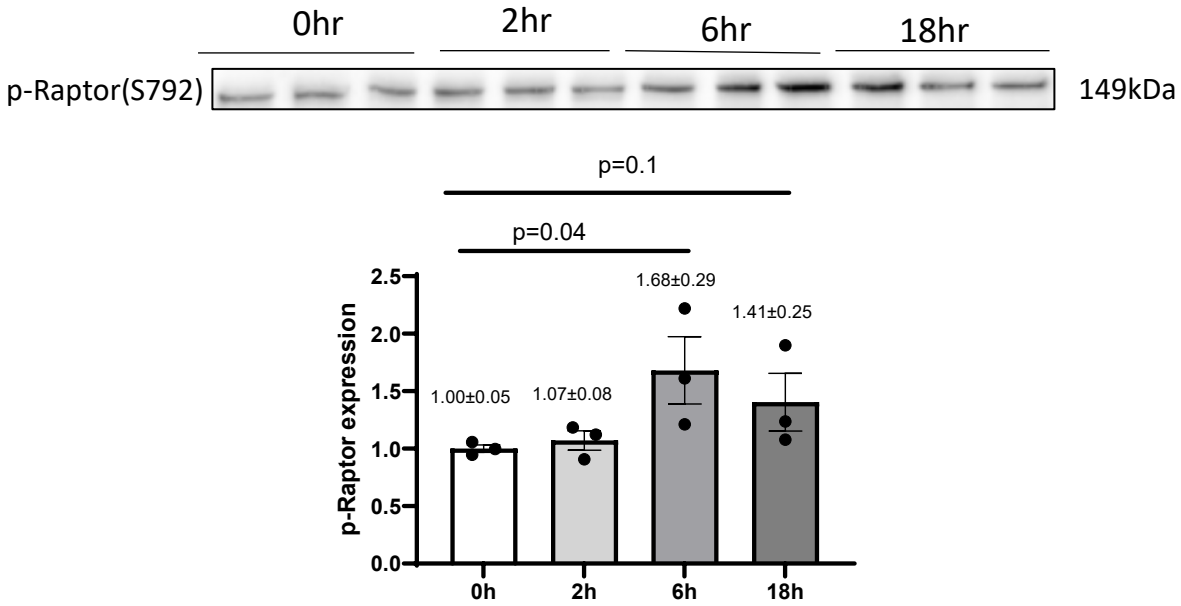


Fig. 3C,D. ALC increases the negative TORC1 effector pRaptor to reduce TORC1 targets pS6K1/p70 & p4E-BP1 in NC.

C



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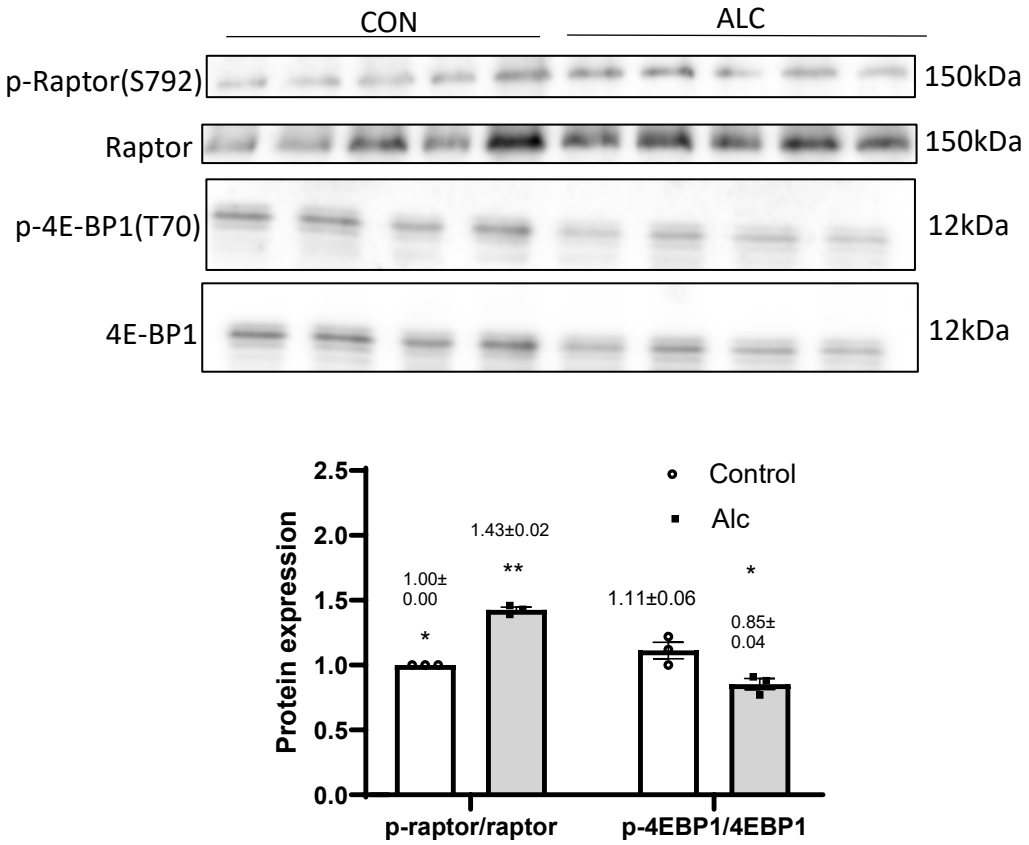


Figure 3E. ALC's effects on TORC1 are selective, and it does not alter the abundance of pRictor, Deptor, pTSC2, or pPKC.

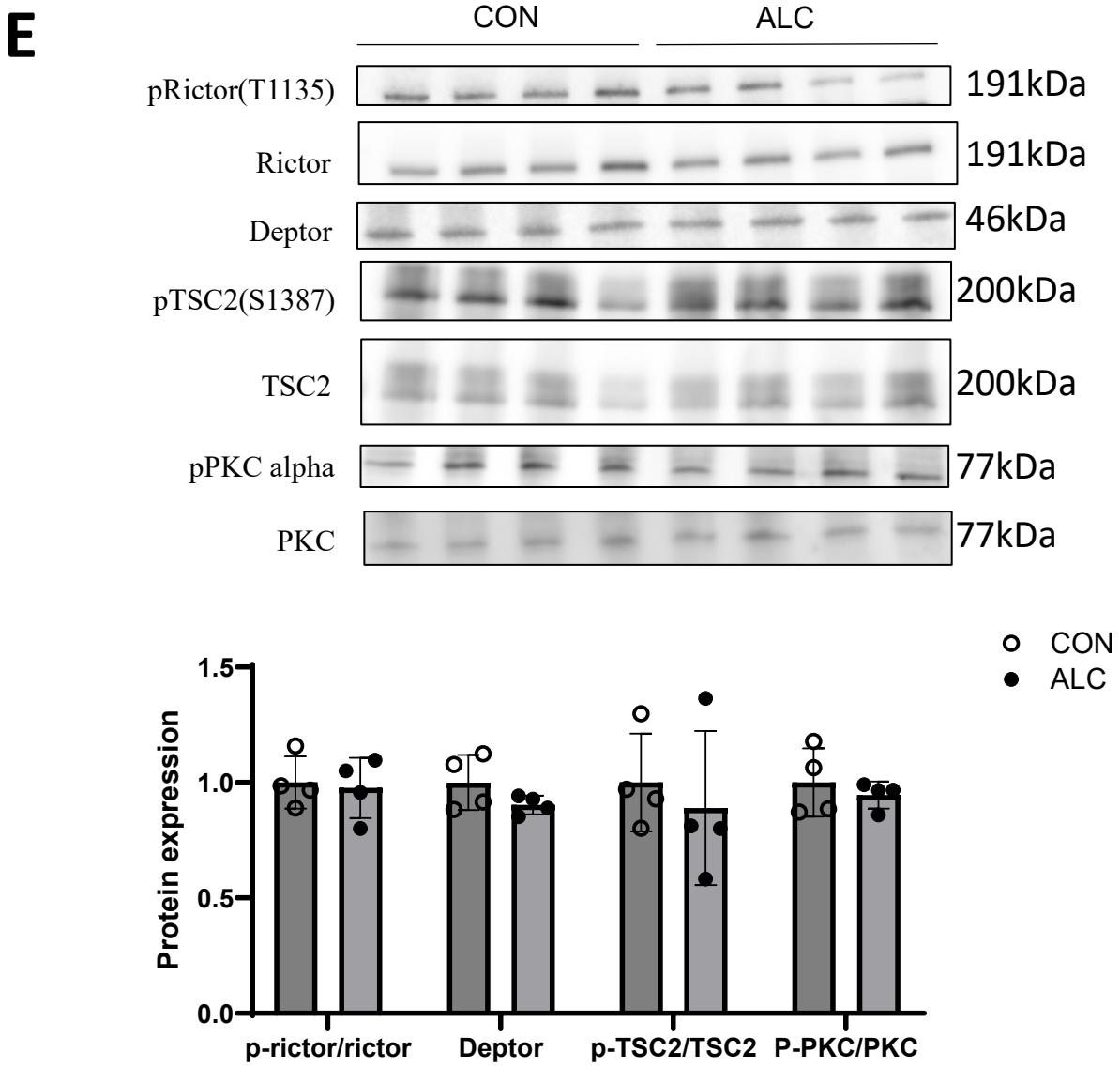
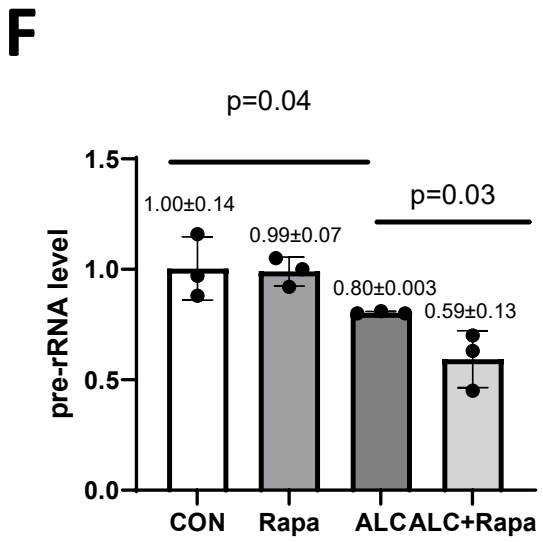
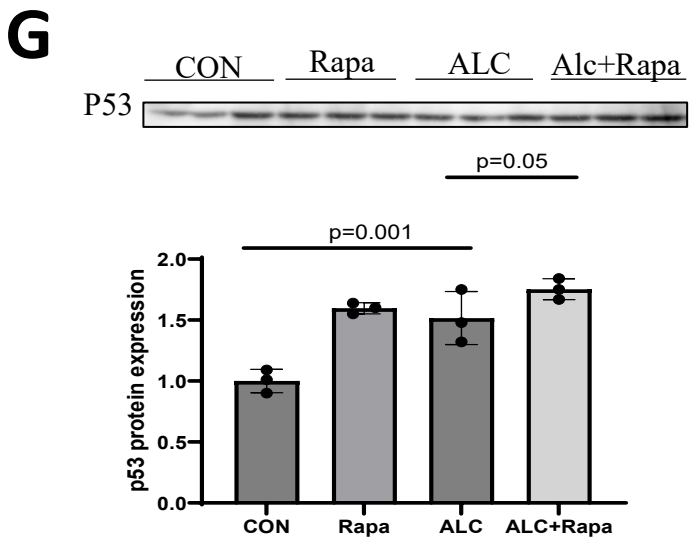


Figure 3F-H. The TORC1 antagonist Rapamycin is sufficient to suppress rRNA synthesis and enhanced p53 stability and apoptosis in ALC NC.





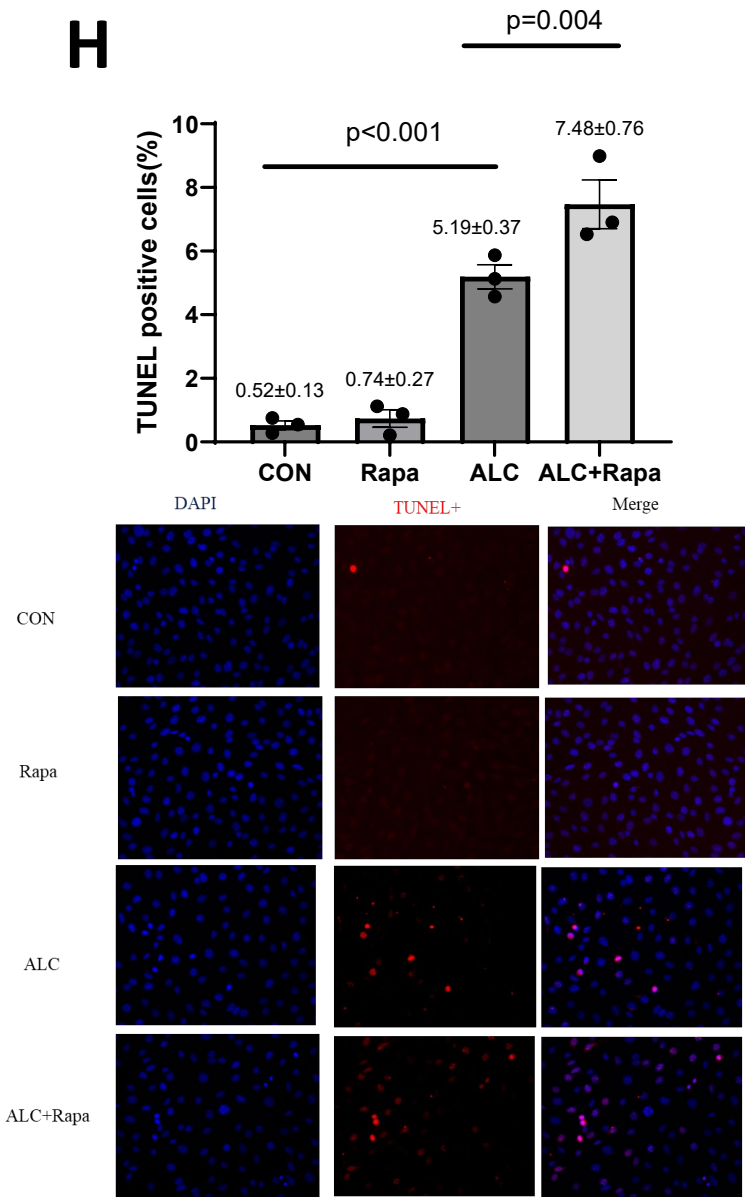
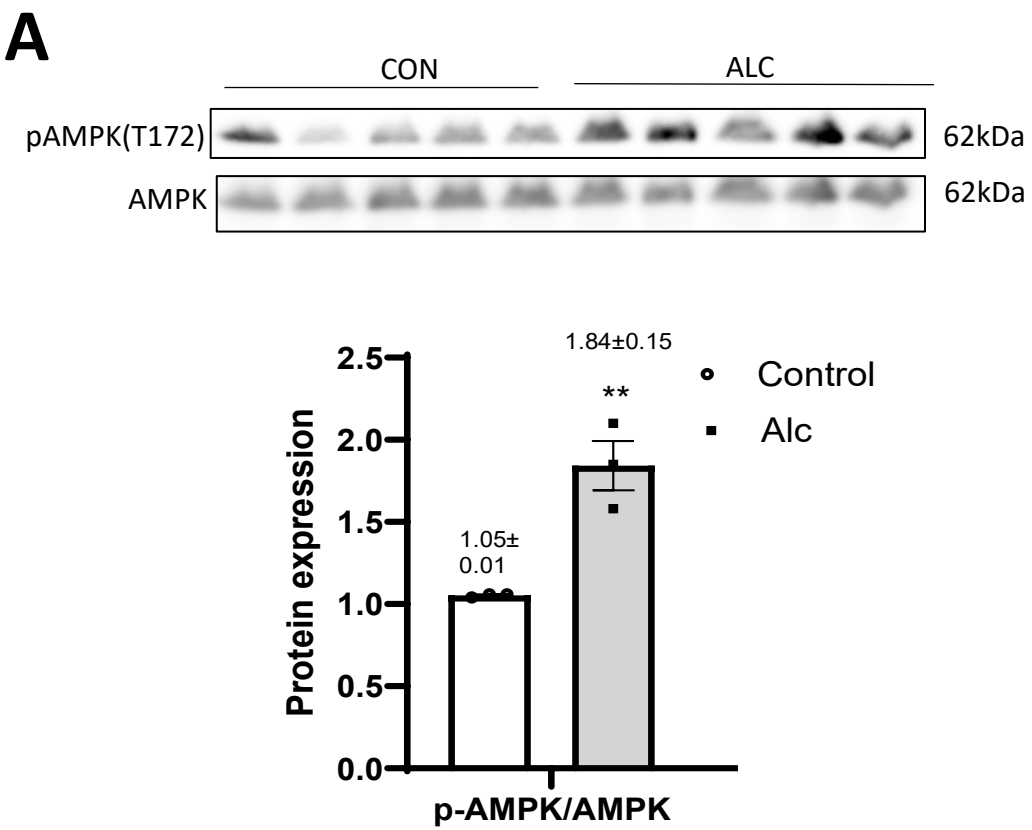


Fig. 4A,B. Alcohol exposure rapidly elevates pAMPK in NC



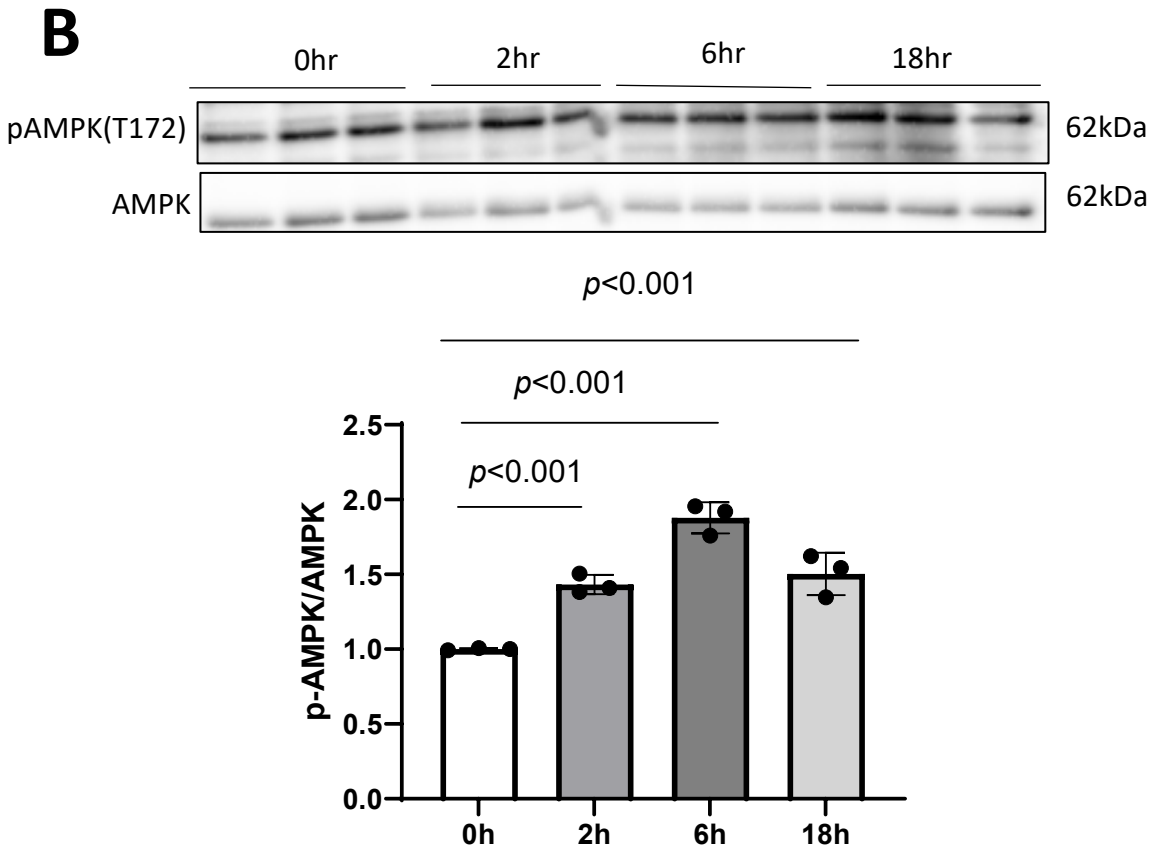
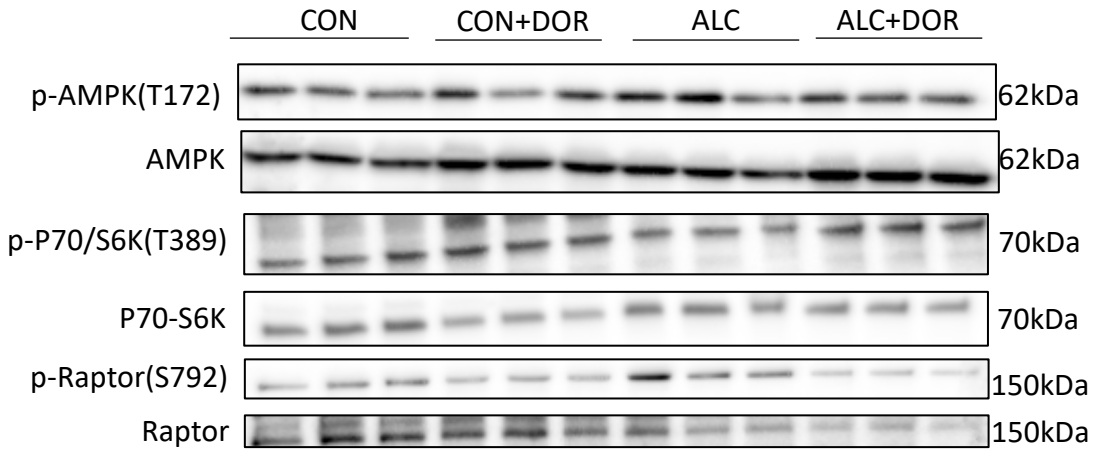
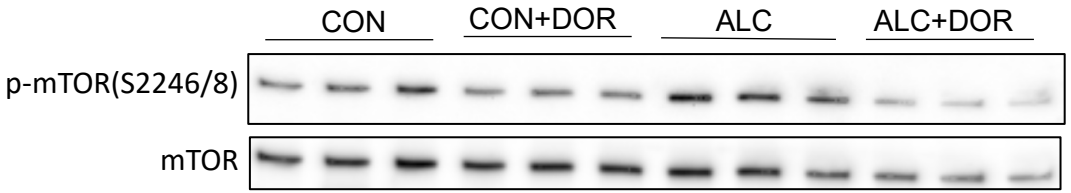
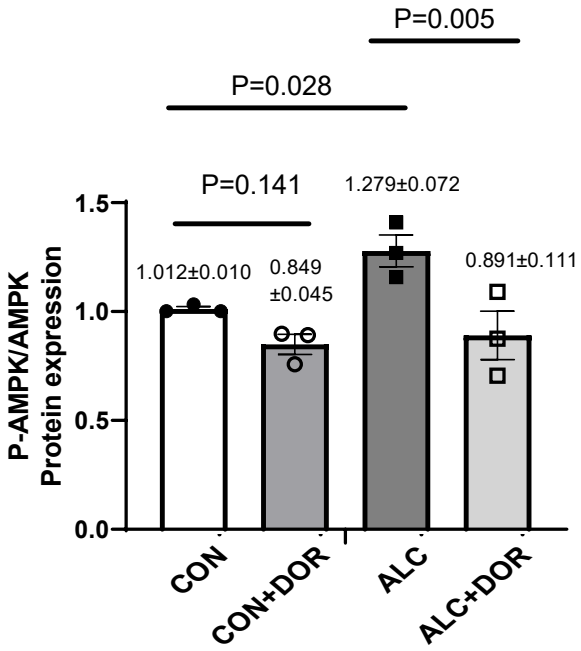
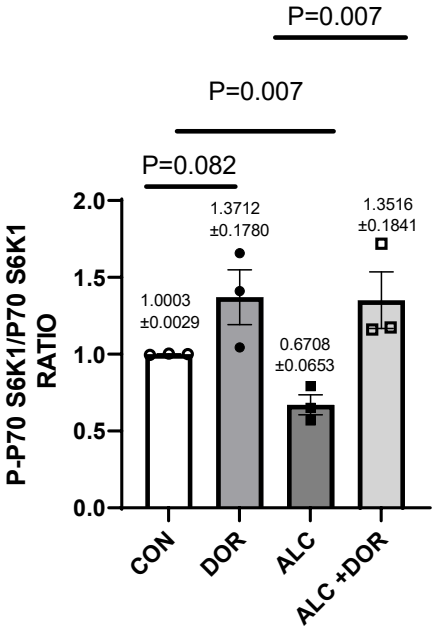


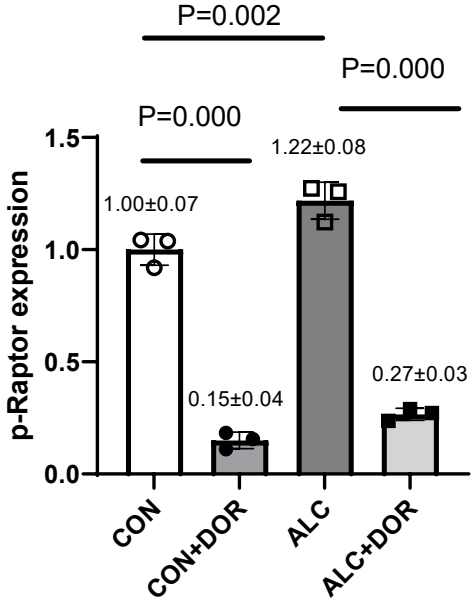
Fig 4C. The AMPK inhibitor Dorsomorphin reduces pAMPK & pRaptor, and increases p-p70/S6K and p-mTOR in ALC NCs











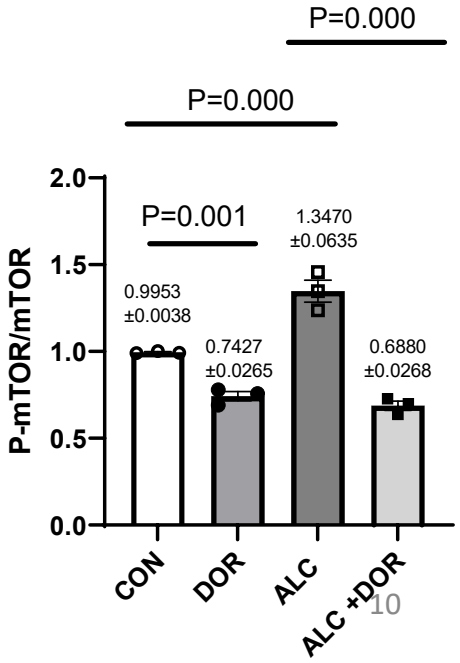
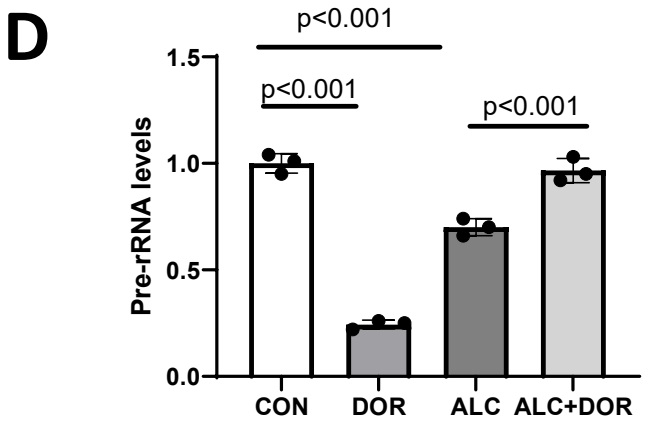
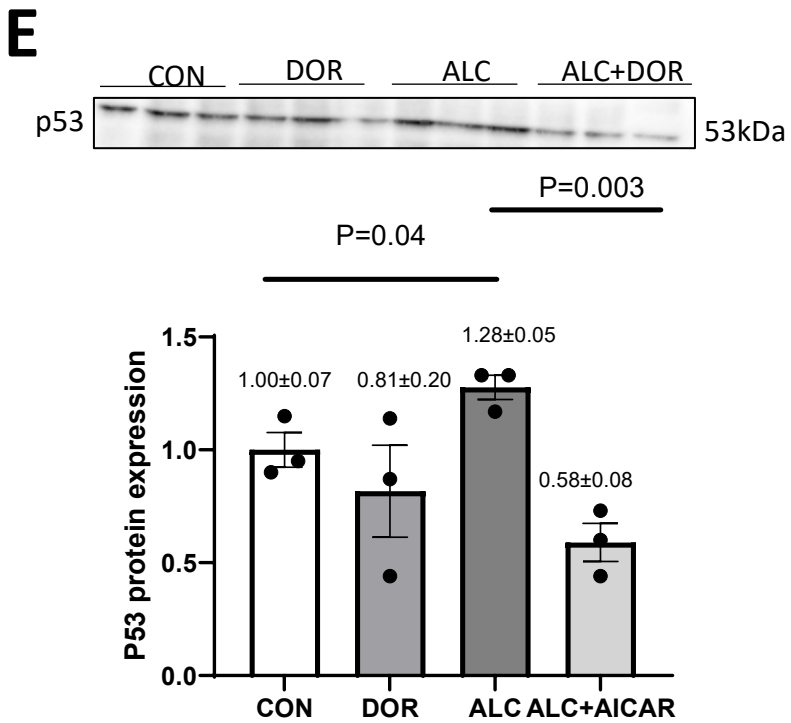
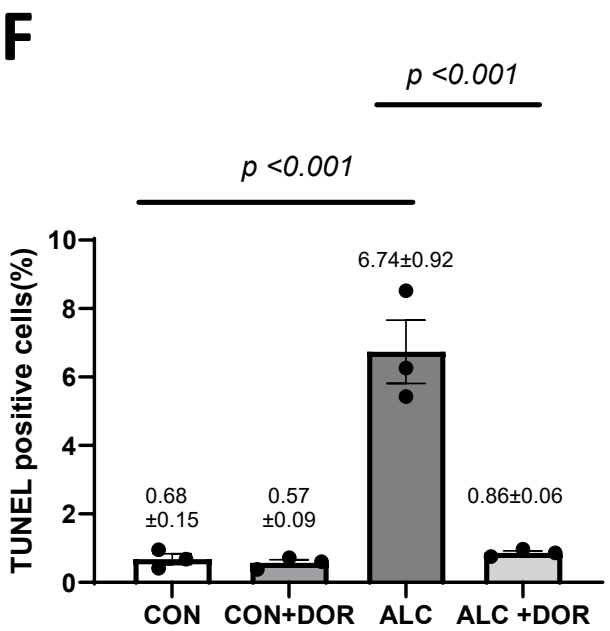


Figure 4D-F. The AMPK inhibitor Dorsomorphin prevents ALC-mediated loss of rRNA synthesis, p53 stabilization, and apoptosis in NC.







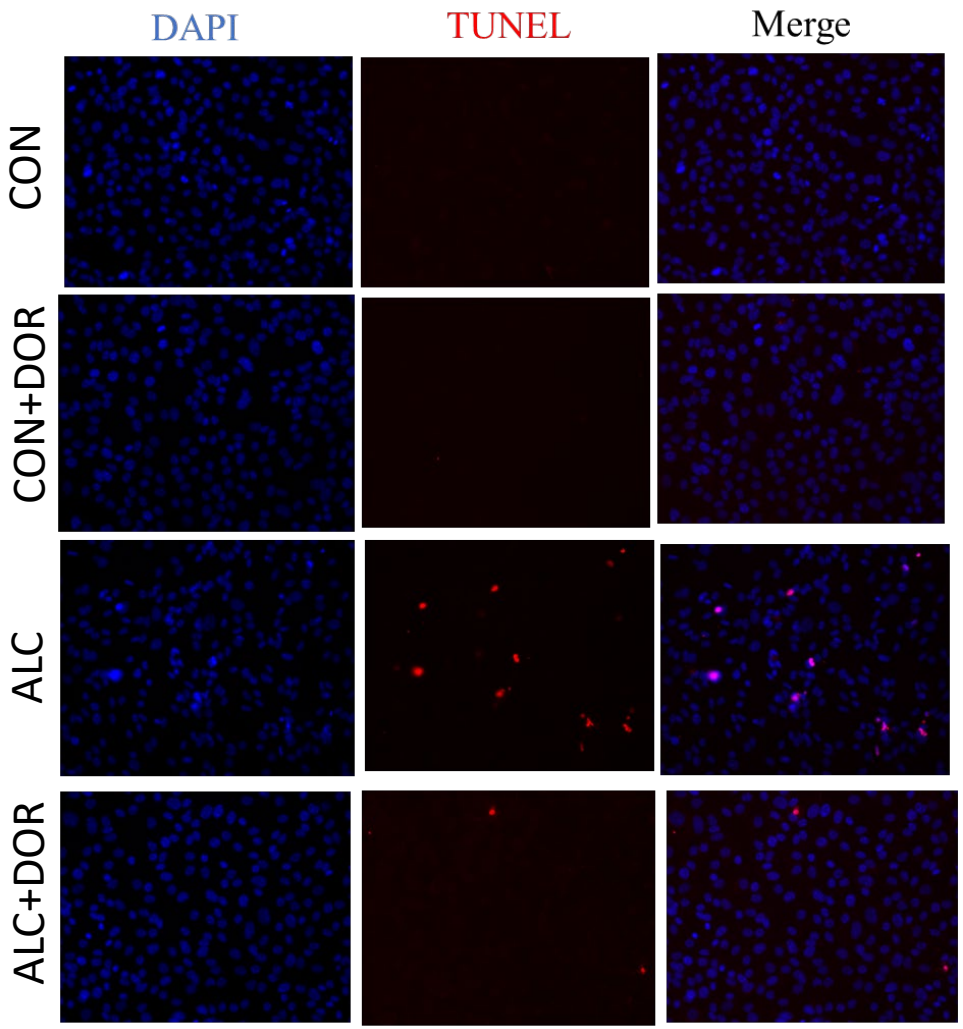
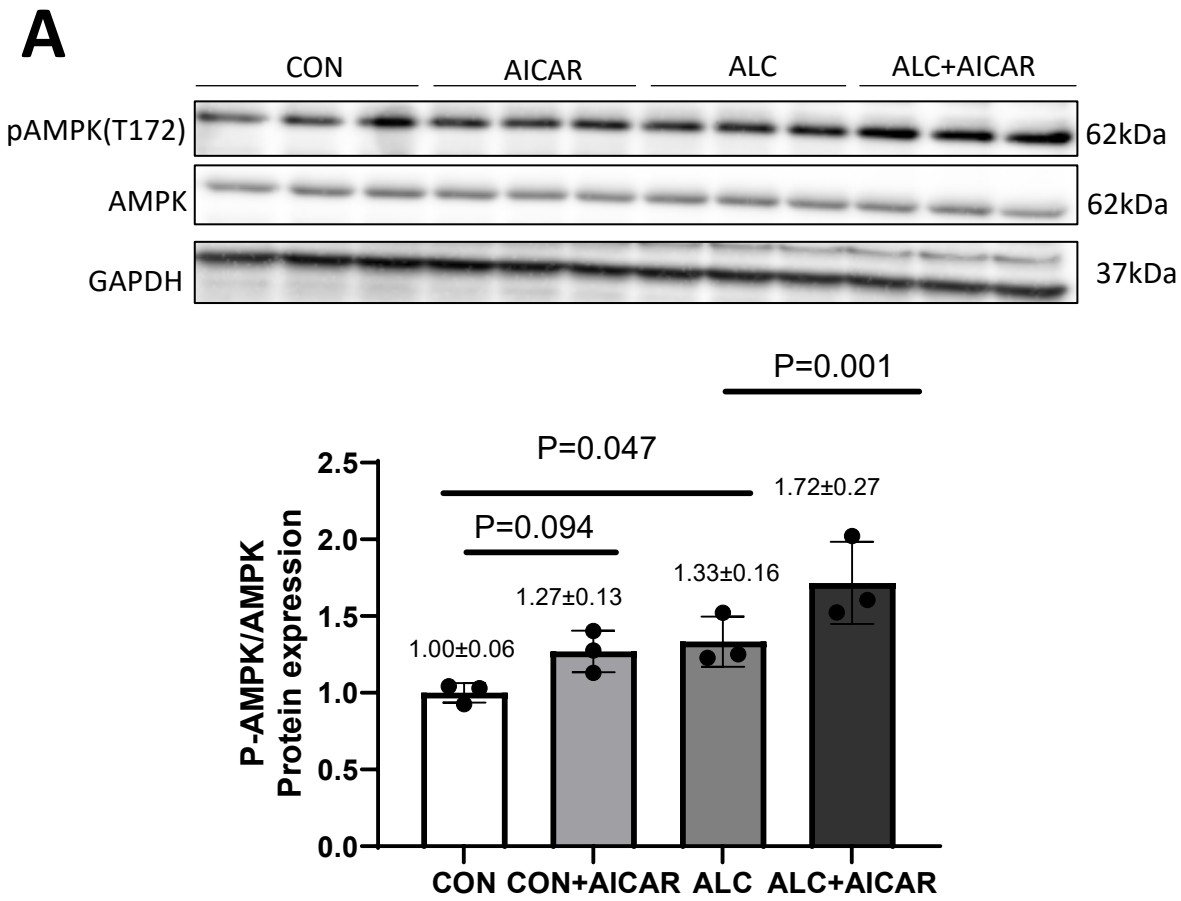


Fig. 5A,B. The AMPK activator AICAR activates pAMPK in normal NC & suppresses p-p70/S6K.



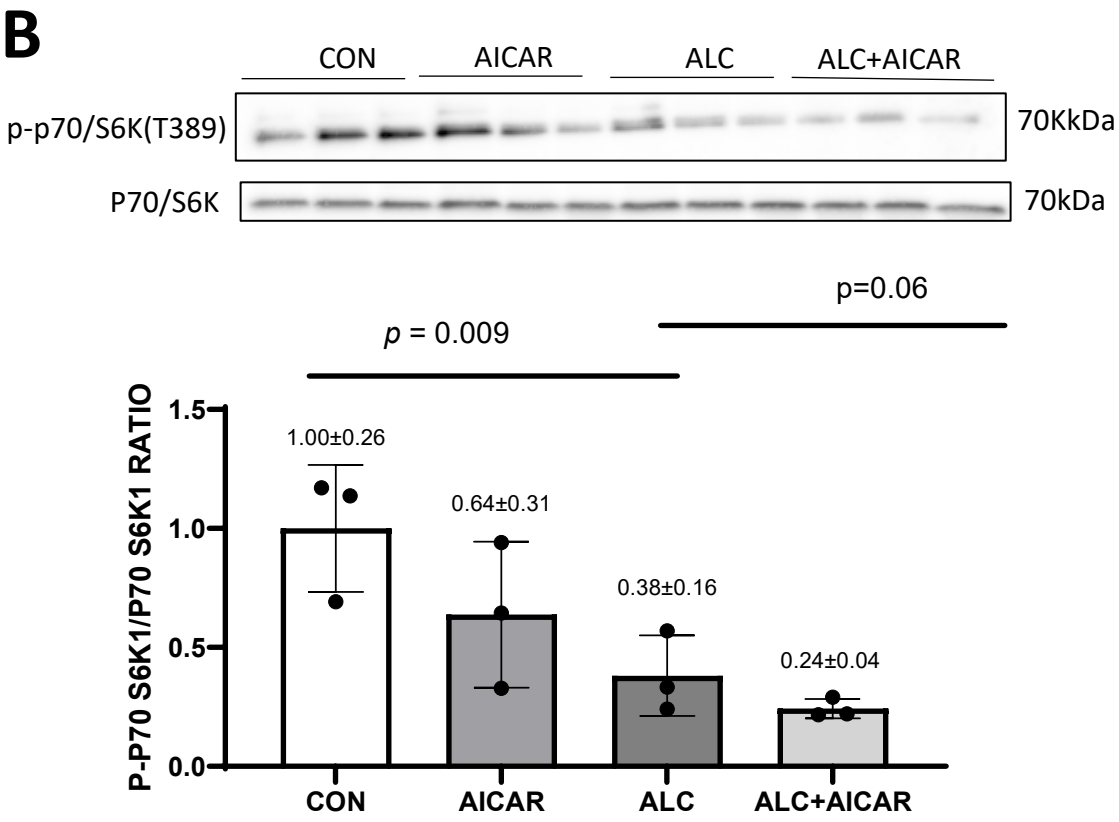
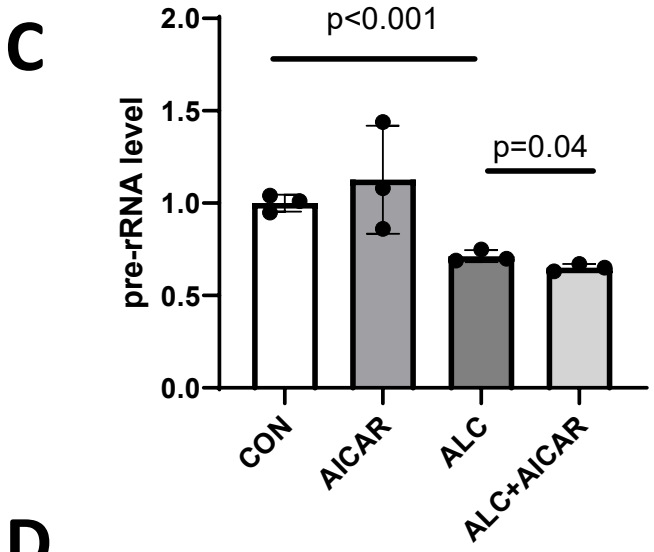
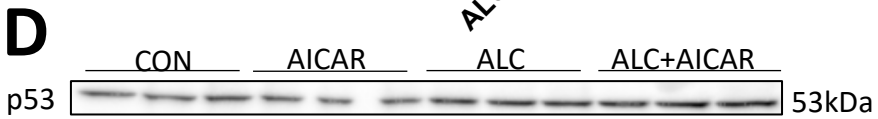
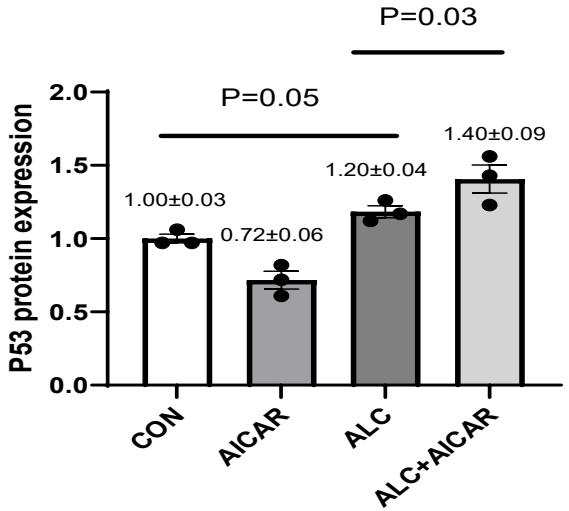
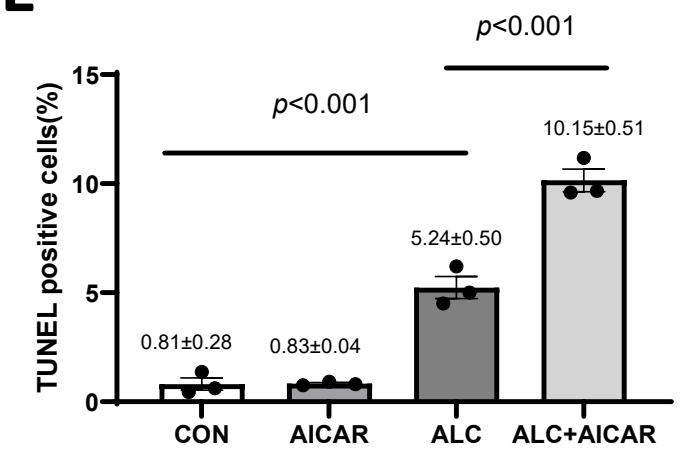
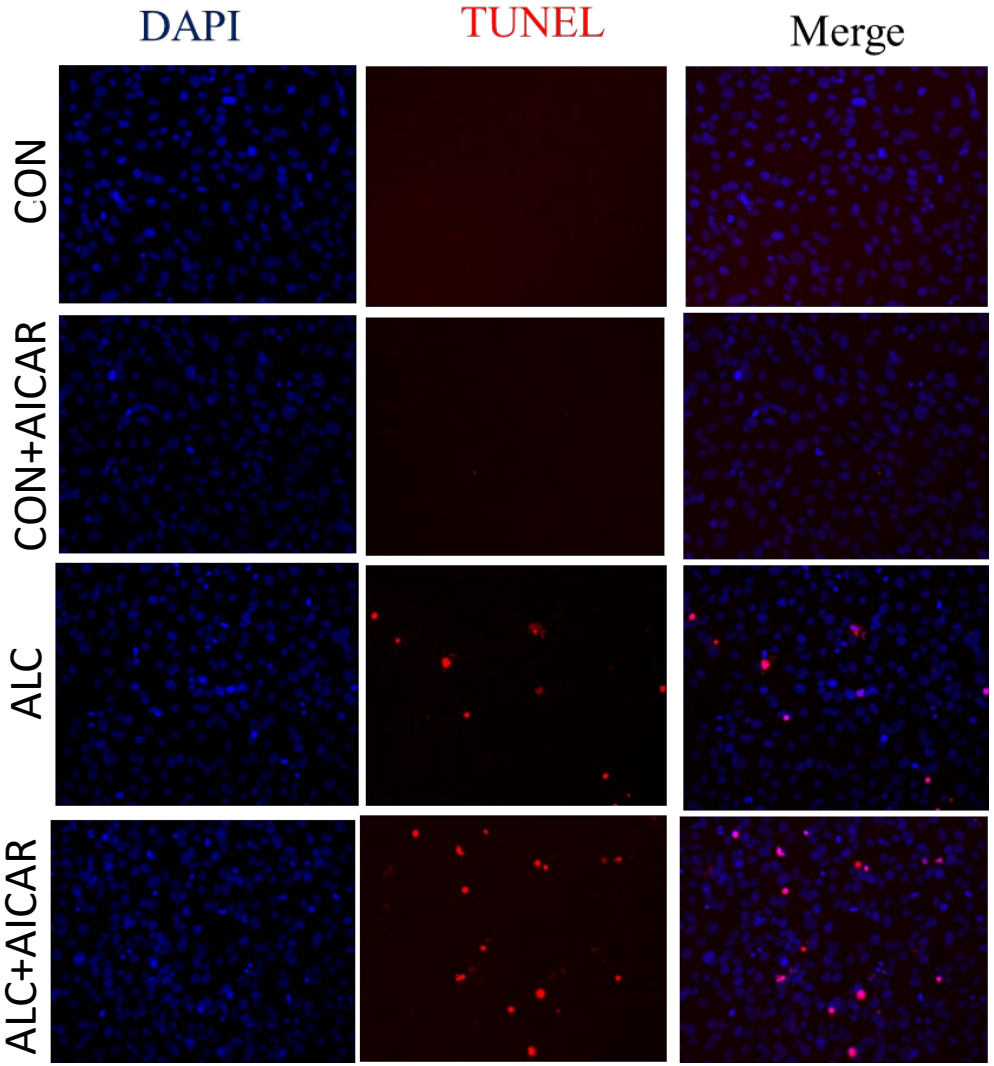



Figure 5C-E. The AMPK activator AICAR suppresses rRNA synthesis and exacerbates ALC-mediated p53 stabilization and apoptosis.