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

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Abstract

Prenatal alcohol exposure is a leading cause of permanent neurodevelopmental disability and can feature distinctive craniofacial deficits that partly originate 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. 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. Alcohol rapidly (<2hr) increased pAMPK, pTSC2, pRaptor, 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 that targeted AMPK, p70/S6K, or TORC1 prevented alcohol's suppression of rRNA synthesis and the induction of p53-stimulated apoptosis. We conclude that alcohol induces ribosome dysbiogenesis and activates their p53/MDM2-mediated apoptosis via its activation of pAMPK, which in turn activates TSC2 and Raptor to suppress the TORC1-p70/S6K-mediated promotion of ribosome biogenesis. This represents a novel mechanism underlying alcohol's neurotoxicity and is consistent with findings that TORC1-p70/S6K networks are critical for cranial NC survival.

1. Introduction

Prenatal alcohol exposure (PAE) is a leading cause of permanent neurodevelopmental disability and features deficits in cognition and executive function [1]. Diagnosis is often initiated by a distinctive craniofacial appearance [2] that has complex origins reflecting both changes in the underlying brain growth [3,4] and direct effects upon the cranial neural crest [5,6], 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 [7]. Neural crest induction begins at neurulation and, shortly thereafter, they 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 neural crest development including their induction, migration, proliferation, and survival [4-6]. We and many others have shown that alcohol causes the apoptotic deletion of neural crest progenitors at a specific phase of their development, at the onset of migration [8-10]. Alcohol interacts with multiple pathways to modulate facial outcome and/or neural crest death including sonic hedgehog, retinoid, oxidative stress, Wnt, and p53, among others [11-17]. However, the regulatory mechanisms governing neural crest sensitivity to alcohol remain elusive. Novel insight into this apoptotic mechanism emerged from our whole exome sequencing of neural crest progenitors, wherein a brief alcohol exposure (2hr, 52mM) rapidly (<6hr) repressed the expression (p_{adj}=10E-47) of 106 nuclear and mitochondrial ribosomal proteins (RP) by 30%-50% [18]. A similar repression of RPs is observed in alcohol-exposed zebrafish [19], headfolds of mouse, [20,21] and chick strains [22] having differential alcohol vulnerability, primary mouse neural stem cells [23] and in the cranial neural crest primary cell line O9-1 [24]. Thus, suppression of RP synthesis is a consistent feature of alcohol-exposed neural crest and

neuroprogenitors across taxons.

Why would a suppression of RPs lead to the apoptosis of cranial neural crest populations? For rapidly proliferating populations such as neural crest, 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 [25], and is seen visually in the nucleosomes, which are the multiple operon sites of this ribosome biogenesis (RBG) [26]. Unsurprisingly, cells have coopted RBG to monitor their internal stress, and RBG is tightly coupled to cellular anabolism and the p53 checkpoint pathway [27,28]. Specifically, under normal conditions RBG is stimulated by the anabolic effector TORC1 via p70/S6K [28]. When energy is limiting, pAMPK phosphorylates the TORC1 components TSC1/2 and Raptor to suppress TORC1 and RBG, thus integrating RBG with cell metabolism. Under stressors that reduce the resources needed for rRNA synthesis, RBG ceases and the nucleoli visually disappear [29,30]. This process, known as “nucleolar stress”, is linked to p53 via the nuclear E3 ubiquitinase MDM2, which under normal conditions targets and destabilizes p53 [31-33]. Under nucleolar stress, reduced rRNA synthesis enables a RPS5-RPL11-5SrRNA complex to bind and inhibit MDM2, thus allowing p53 to become transcriptionally active [31-33]. The importance of RBG for neural crest is evidenced in the genetic disorders known as ribosomopathies, in which loss-of-function in AMPK-TORC1-p70/S6K signaling, or in components of RBG such as rRNA, RPs, or the ribosome assembly machinery, cause p53-mediated neural crest losses and facial deficits that share similarities with those of PAE (i.e. Treacher-Collins syndrome, Diamond-Blackfan anemia) [34-36].

We recently reported that alcohol exposure impairs RBG to induce nucleolar stress in

early cranial neural crest progenitors. Alcohol exposure causes the rapid cessation of rRNA synthesis and dissolution of nucleolar structures in response to pharmacologically relevant alcohol exposures (20-80mM for 2hr) [24]. This is followed by the stabilization of nuclear p53 and their subsequent p53-mediated apoptosis [17,24]. Haploinsufficiency of RPL5, RPL11, or RPS3A using a morpholino approach synergized with alcohol to cause craniofacial deficits in a zebrafish model of PAE [18,24], and transfection with MDM2 or loss-of-function p53 blocked these effects of alcohol [24]. 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) [37,38]. 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 [37]. 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 [37-40]. We find it has alcohol responses identical to those of primary avian or mouse NCs [24]. 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 were tested within 18hr.

2.2. Alcohol Exposure

Cells were treated with USP grade ethanol (Koptec, King of Prussia, PA) at 80mM; we previously found this is the EC50 for the alcohol-mediated dissolution of nucleolar structures in O9-1 cells [24]. In these culture conditions, we find that the alcohol concentrations drop to 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. Total RNA (1μg) 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 the 18S and 5.8S rRNA of newly transcribed 47S rRNA [23,24]. The primers (IDT, Coralville, IA) for ITS-1 were forward: 5′-CCGGCTTGCCCGATTT-3′, reverse: 5′-GCCAGCAGGAACGAAACG-3′ and for β2 microglobulin were forward: 5′-TTCACCCCCACTGAGACTGAT-3′; reverse: 5′-GTCTTGGGCTCGGCCATA-3′. Relative

abundance was calculated using the $2^{-\Delta\Delta CT}$ method and was normalized to β2 microglobulin. qPCR methodology adhered to the MIQE standards [41].

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 $NaVO_3$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride; all from Sigma). Total protein was separated on 10% SDS-PAGE reducing gels for proteins ≤150kDa, or 4%-20% detergent gels for those ≥150kDa (BioRad). The resolved proteins were transferred to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). Primary antibodies directed against p-AMPK(Thr172) (#2535), AMPK (#2793), p-mTOR(Ser2446/2448) (#2971), mTOR (#4517), p-Raptor(Ser792) (#2083), Raptor (#2280), p-p70/S6K1(Thr389) (#9234), p70/S6K1 (#9202), pRictor(Thr1135) (#3806), Rictor (#2140), pTSC2(Ser1387) (#5584), TSC2 (#4308), PKCα (#2056), p4E-BP1(Thr70) (#9455), and 4E-BP1(#9452) were all used at 1:1000 and were from Cell Signaling (Danvers MA). Antibodies directed against p53 (#ab26, 1:500), Deptor (#191841, 1:200) and pPKCα(Ser657) (#ab180848) were from Abcam (Cambridge, England, UK). Anti-GAPDH (#G8795, 1:2500) was from Sigma. The secondary antibodies were goat anti-rabbit (#4010-05, 1:5000) and anti-mouse immunoglobulin (#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; 42) and Lipo-3000 (#L3000001; Invitrogen) according to the manufacturer's instructions. This construct contains four gain-of-function mutations (T388E, S410D, S417D, S423D) that confer rapamycin-resistance and constitual activity (ca-S6K; 42). Transfected 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 TUNEL assay (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), the TORC1 inhibitor rapamycin (1 nM; all from Selleck Chemicals, Houston TX), or the TORC1 activator L-leucine (250 μM, Sigma). For all, 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 comparisons of two groups and one-way ANOVA for multiple comparisons. $P < 0.05$ was considered significant. Unless otherwise indicated, all results are presented as the mean ± SD of

3 independent samples per group.

3. Results

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

As we showed previously [24], a time course reveals that exposure to 80mM alcohol suppressed *de novo* rRNA synthesis by 1hr following exposure (CON 100 ± 11.7%, ALC 63.5 ± 4.8%; $p<0.001$; **Figure 1A**), and this reduction persisted for at least 4hr to 8hr thereafter. This was followed by a dissolution of nucleolar structures that was visualized using antibodies directed against the RNA polymerase I transcription factor UBF (**Figure 1B**) [24,29]. The proapoptotic protein p53, which responds to nucleolar stress via MDM2 [31-33] was stabilized in these cells within 2hr of exposure, when its content increased 70 ± 11% ($p<0.001$). We showed previously [37] that this largely represents nuclear protein and its elevation persisted for at least 12hr (CON 100 ± 42%, ALC 191 ± 14%; $p<0.001$; **Figure 1C**), whether normalized to GAPDH (upper blots) or total protein (lower blot). By 16hr, the alcohol-exposed NC underwent apoptosis (CON 0.97 ± 0.23%, ALC 10.94 ± 1.24%; $p<0.001$; **Figure 1D**), and we showed previously that, as in other models [16,17,23], this apoptosis is p53-dependent because it is prevented by transfection with dominant-negative p53 [24].

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

To gain insight into the mechanism by which alcohol induced nucleolar stress in neural crest, we evaluated its impact upon a primary effector of RBG, p70/S6K [28]. Alcohol exposure reduced

the abundance of p70/S6K in a complex manner. With respect to total p70/S6K protein, its content was 69 ± 6% (p<0.001) of CON values within 2hr of alcohol exposure, and these reductions persisted through 6hr and were normalized by 12hr post-exposure (**Figure 2A**). This reduction was mediated, in part, at the transcriptional level because the abundance of transcripts encoding p70/S6K was 89.7% of CON (p_{adj}=4.28E-12) as determined by whole exome sequencing. With respect to its 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 they dropped to 57 ± 1% at 12hr (p<0.001) and 59 ± 7% at 18hr (p=0.004) post-exposure. However, because of the absolute decline in p70/S6K, this represented a 30-40% decline in the abundance of activated p70/S6K within 2hr of the alcohol exposure.

To test the functional involvement of this p70/S6K loss, we transfected NC with a constitutively active (ca) version of p70/S6K (caS6K), which contains four gain-of-function mutations that confer rapamycin-resistance (42). As expected, the caS6K construct countered the alcohol-mediated reduction in p-p70/S6K(T389) (ALC, 55 ± 7%, ALC+caS6K, 83 ± 12%, p=0.022; **Figure 2B**) to levels not different from CON (1.00 ± 0.16, p>0.10). It restored *de novo* rRNA synthesis in alcohol-exposed cells (ALC 49 ± 5%, ALC+caS6K 90 ± 3%, p<0.001; **Figure 2C**), abrogated alcohol's activation of p53 (ALC, 155 ± 29%, ALC+caS6K, 106 ± 14%; p=0.05; **Figure 2D**), and prevented their apoptosis (**Figure 2E,** ALC, 2.70 ± 0.45%, ALC+caS6K, 0.52± 0.11%; p <0.001). In the controls, caS6K unexpectedly reduced rRNA synthesis (CON, 100 ± 6%, CON+caS6K, 54 ± 1%, p<0.001), but this had no effect on p53 content or apoptosis. We conclude that alcohol rapidly suppressed p70/S6K and that this contributes to the loss of RBG.

3.3. Alcohol activates the upstream TORC1 repressors pTSC2 and pRaptor to reduce

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 core component protein mTOR by kinases including Akt, AMPK, and p70/S6K itself [27]. 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. Although these two sites could not be distinguished, they are under quite different regulation; S2446 is targeted by AMPK to reduce TORC1 activity, whereas S2448 is targeted by both Akt and p70/S6K to increase TORC1 signaling [27]; however, increased TORC1 signaling was inconsistent with the downstream reduction in p70/S6K. To disentangle whether this represented an activation or repression of TORC1, we evaluated TSC2 and Raptor, which act immediately upstream as AMPK-dependent negative regulators of TORC1 [43,44]. Alcohol increased the content of pTSC2(S1387) within 2hr of exposure (CON, 100 ± 1%, ALC. 144 ± 25%; p=0.05; **Figure 3B**) and increased p-Raptor(S792) by 6hr (CON, 100 ± 5%, ALC. 168 ± 29%; p=0.04); TSC2 was largely normalized by 6hr, whereas pRaptor remained elevated for at least 12hr (ALC 12hr, 143 ± 2%, p<0.001 vs CON). As further evidence that this represented a reduction in TORC1 activity, we found a reduction in a second TORC1 target p4E-BP1(T70) (CON, 111 ± 6%, ALC 85 ± 4%, p=0.027; **Figure 3C**), which is otherwise activated by TORC1 to increase protein translation in concert with p70/S6K's stimulation of RBG [27,28]. We also explored alcohol's impact on effectors of TORC2 and found no differences with respect to the abundance of pRictor(T1135), Deptor, pTSC2(S1387), or pPKCα(S657), suggesting alcohol's suppression of TORC1 was selective in these neural crest cells (**Figure 3D**).

To explore these findings further, we tested if targeted inhibition of the TORC1 signaling

pathway was sufficient to suppress RBG and induce NC apoptosis. Pretreatment with rapamycin (Rapa), a small molecule inhibitor of TORC1 [45], prior to alcohol exposure further suppressed rRNA synthesis (ALC, 80 ± 0.3%, ALC+Rapa, 59 ± 13%; p=0.03; **Figure 4A**), and aggravated p53 stabilization (ALC, 151 ± 21%, ALC+Rapa, 175 ± 8%; p=0.05; **Figure 4B**) and apoptosis (ALC, 5.19 ±0.37%, ALC+Rapa, 7.48± 0.76%; p=0.004; **Figure 4C**). Interestingly, rapamycin increased the p53 content in control cells but did not suppress their rRNA synthesis or cause apoptosis, indicating that TORC1 inhibition was insufficient, in of itself, to suppress RBG and induce apoptosis in these cells.

We further tested TORC1 contributions functionally by providing the TORC1 activator L-leucine just prior to the alcohol-exposure. We found that supplemental L-leucine (at 250% of normal cell culture levels) enhanced rRNA synthesis in alcohol-exposed cells (ALC, 77 ± 6%, ALC+Leu, 143 ± 26%, p=0.001; **Figure 4D**), and abrogated the loss of total p70/S6K protein (p=0.004) but did not further affect p-p70/S6K content (**Figure 4E**). It also normalized p53 levels (p<0.001; **Figure 4F**) and prevented their alcohol-induced apoptosis (p<0.001; **Figure 4G**). In CON, it increased p-p70/S6K levels (p=0.005) and rRNA synthesis (CON, 100 ± 5%, CON+Leu, 159 ± 15%, p=0.002). Taken together, these data suggested that acute alcohol rapidly repressed TORC1 activity and this might be mediated through the activation of TSC1 and Raptor.

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

The canonical repressor of TORC1 activity is the AMP-dependent kinase (AMPK), which inhibits TORC1 via its phosphorylation of TSC2 and pRaptor [43,44]. Consistent with its actions

upon TSC2, Raptor, TORC1, and p70/S6K, alcohol exposure caused a rapid elevation in the activated form of AMPK, pAMPK(Thr172), within 2hr of exposure (CON, 100 ± 1%, ALC 143 ± 6%, $p<0.001$; **Figure 5A**). The 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 treated cells with the AMPK inhibitor dorsomorphin prior to their alcohol exposure [43,44]. Dorsomorphin attenuated alcohol's activation of pAMPK(T172) (ALC, 128 ± 7%, ALC+DOR, 89 ± 11%, $p=0.005$) and normalized the content of downstream targets pRaptor(S792) (ALC, 122 ± 8%, ALC±DOR, 27 ± 3, $p<0.001$), p-mTOR(S2246/8) (ALC 135 ± 6%, ALC+DOR, 69 ± 3%, $p<0.001$), and p-p70/S6K(T389) (ALC, 67 ± 6%, ALC+DOR, 135 ± 18%, $p=0.007$; **Figure 5B**). The AMPK inhibitor also normalized *de novo* rRNA synthesis (ALC, 70 ± 4%, ALC±DOR, 97 ± 6%, $p<0.001$; **Figure 5C**), and prevented the stabilization of p53 (ALC, 128 ± 5%, ALC±DOR, 58 ± 8%; $p=0.003$; **Figure 5D**) and subsequent apoptosis in response to alcohol (ALC 6.74 ± 0.92%, ALC+DOR 0.86 ± 0.06%; $p<0.001$; **Figure 5E**). These data suggested that alcohol caused neural crest apoptosis through the AMPK-mediated inhibition of TORC1 and p70/S6K to suppress their RBG.

We then asked if inappropriate activation of pAMPK was sufficient to invoke nucleolar stress and p53-mediated apoptosis in neural crest. Using the AMP mimetic and AMPK activator AICAR [43,44], we found that in otherwise normal cells it non-significantly elevated pAMPK(T172) (CON, 100 ± 6%, CON+AICAR, 127 ± 13%, $p=0.094$; **Figure 6A**) and reduced p-p70/S6K(T389) (CON, 100 ± 26%, CON+AICAR, 64 ± 31%, $p>0.1$; **Figure 6B**). However, it was not sufficient to suppress their rRNA synthesis nor did it stabilize p53 or cause their

apoptosis (**Figure 6C-E**). In cells exposed to alcohol, it further elevated pAMPK(T172) (ALC, 133 ± 16%, ALC+AICAR, 172 ± 27%, p=0.001) and trended to further reduce p-p70/S6K(T389) content (ALC, 38 ± 16%, ALC+AICAR, 24 ± 4%, p=0.06). However, 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 neural crest.

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 ribosome biogenesis, induce nucleolar stress, and effect their p53-mediated apoptosis. Moreover, this suppression of p70/S6K was driven by alcohol's upstream activation of pAMPK. Counteracting these changes through 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 within 1hr and increases in both pAMPK and p53 within 2hr post-exposure, suggesting these represent primary targets of alcohol. We reported previously that pharmacologically relevant alcohol exposures (20-80mM) suppress RBG and induce nucleolar stress in cranial neural crest cells, leading to their p53/MDM2 mediated apoptosis. The rapidity of pAMPK's activation in response to alcohol, and the ability of interventions targeting AMPK-TORC1-p70/S6K gain- and loss- of-function to alter alcohol's effects on RBG and p53-mediated apoptosis, suggests that AMPK

activation and its downstream suppression of TORC1-p70/S6K are primary effectors of this alcohol-induced apoptosis. These actions of alcohol upon the AMPK-TORC1-p70/S6K pathway to instigate neural crest apoptosis are summarized in **Figure 7**.

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 [46], as TORC1 can operate downstream from PDGFR. Because that study did not directly access TORC1 activity, alcohol's potential impact on TORC1 in that model is unclear. Acute alcohol exposures reduce TORC1 signaling in adult cerebral cortex [47], similar to its effects here. Using the same antibody reagent as used here (#2971, Cell Signaling), chronic exposure reduced p-mTOR in fetal hippocampus [48]; however, this was accompanied by elevated p-p70/S6K and p4E-BP1, along with elevated Deptor and Rictor but not Raptor, outcomes distinct from those reported here. It is possible that chronic alcohol exposure led to adaptive or compensatory changes in response to reduced p-mTOR or may have dysregulated aspects of that pathway [48]. Our findings are consistent, however, with the increased ULK/ATG signals in alcohol-exposed cells [49,50], as autophagy also increases under TORC1 suppression, and this has been posited to reflect a protective response to alcohol [49,50]; we did not assess autophagy here. Acute alcohol (18-24hr) also suppresses the phosphorylation of the downstream TORC1 targets p70/S6K and 4E-BP1 in SH-SY5Y neuroblastoma cells [51] and C2C12 myocytes [52] and promoted apoptosis in that neuronal model [51]. That TORC1 signaling is indispensable for neural crest is highlighted by studies in which its modulation by genetic [53] or dietary means [54] disrupts

craniofacial morphogenesis and alters the facial appearance. Taken together, the critical requirement of neural crest for high rates of RBG to sustain their equally high proliferative rate prior to their migration [25,55,56], and the requirement for TORC1 to sustain p70/S6K activation and RBG [28], could explain how alcohol's suppression of TORC1 and p70/S6K 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 [27,43,44], alcohol's activation of pAMPK and its targets pTSC2 and pRaptor suggest that alcohol reduced RBG via repression of TORC1 signaling. This was further endorsed in that treatment of ALC neural crest with the AMPK antagonist dorsomorphin reversed alcohol's impact and normalized pAMPK, pS6K, p-mTOR, and pRaptor, and prevented both nucleolar stress and apoptosis. That AMPK inhibition blocked alcohol's adverse effects indicates that pAMPK participates in their apoptosis. However, that neither rapamycin nor AICAR were sufficient to suppress rRNA synthesis or cause apoptosis in otherwise healthy neural crest suggests that signals in addition to pAMPK and TORC1 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 [57]. For example, we have previously shown that in cranial neural crest alcohol initiates a G-protein-mediated intracellular calcium transient that activates CaMKII and destabilizes nuclear β-catenin to trigger their apoptosis [15,58-60]. Alcohol also has been shown to modulate facial outcome through signals involving sonic hedgehog, retinoids, and oxidative stress, among others [5,6,11-15]. Studies are underway to elucidate these additional contributors that further destabilize these neural crest cells and tip them into an apoptotic fate.

Alcohol has been shown to increase pAMPK in multiple models including neurons of the hippocampus [61], prefrontal cortex [62], isolated cardiac and myoblast cells [63-65], and hepatocytes [66]. Blockade of AMPK activation prevents neuronal cell death in models of zinc-induced toxicity [67] and Parkinson's disease [68]. 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 mechanisms including binding to elevated AMP, carbohydrate binding motifs and, of relevance to this model, pCAMKK2 in response to intracellular calcium signals [43,44,69]. There is debate whether AMPK activation in response to alcohol represents a beneficial or deleterious effect [70], 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 [43,44]. This latter may be especially detrimental to cranial neural crest given their absolute dependence upon RBG as evidenced in the genetic disorders known as ribosomopathies, in which RBG loss-of-function causes p53-mediated neural crest losses and facial deficits that parallel those of PAE (i.e. Treacher-Collins, Diamond-Blackfan Anemia) [34-36]. 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 neural crest 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.

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Author Contributions

YH - Conceptualization, methodology, formal analysis, investigation, writing original draft, writing edit and review, visualization; GRF - Conceptualization, methodology, investigation, writing edit and review; SMS - Conceptualization, writing original draft, writing edit and review, visualization, administration, funding.

References

1. Hoyme HE, Kalberg WO, Elliott AJ, Blankenship J, Buckley D, Marias AS, et al. 2016. Updated clinical guidelines for diagnosing fetal alcohol spectrum disorders. Pediatrics 138:e201542568.
2. Suttie M, Foroud T, Wetherill L, Jacobson JL, Molteno CD, Meintjes EM, Hoyme HE, Khaole N, Robinson LK, Riley EP, Jacobson SW, Hammond P. 2013. Facial dysmorphism across the fetal alcohol spectrum. Pediatrics. 131:e779-88.
3. Foroud T, Wetherill L, Vinci-Booher S, Moore ES, Ward RE, Hoyme HE, Robinson LK, Rogers J, Meintjes EM, Molteno CD, Jacobson JL, Jacobson SW. 2012. Relation over time between facial measurements and cognitive outcomes in fetal alcohol-exposed children. Alcohol Clin Exp Res 36:1634-1646.
4. Lipinski RJ, Hammond P, O'Leary-Moore SK, Ament JJ, Pecevich SJ, Jiang Y, Budin F, Parnell SE, Suttie M, Godin EA, Everson JL, Dehart DB, Oguz I, Holloway HT, Styner MA, Johnson GA, Sulik KK. 2012. Ethanol-induced face-brain dysmorphology patterns are correlative and exposure-stage dependent. PLoS One. 7(8):e43067.
5. Smith SM, Garic A, Flentke GR, Berres ME. 2014. Neural crest development in fetal alcohol syndrome. Birth Defects Res C Embryo Today. 102:210-20.
6. Chen SY, Kannan M. 2023. Neural crest cells and fetal alcohol spectrum disorders: Mechanisms and potential targets for prevention. Pharmacol Res. 194:106855.
7. Bhatt S, Diaz R, Trainor PA. 2013. Signals and switches in mammalian neural crest cell differentiation. Cold Spring Harb Perspect Biol. 5(2):a008326.
8. Cartwright MM, Tessmer LA, Smith SM. 1998. Ethanol-induced neural crest apoptosis is coincident with their endogenous death but is mechanistically distinct. Alcohol Clin Exp Res 22:142-149.
9. Dunty WC Jr, Chen SY, Zucker RM, Dehart DB, Sulik KK. 2001. Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. Alcohol Clin Exp Res. 36:1340-1354.
10. Fan H, Li Y, Yuan F, Lu L, Liu J, Feng W, Zhang HG, Chen SY. 2022. Up-regulation of microRNA-34a mediates ethanol-induced impairment of neural crest cell migration in vitro and in zebrafish embryos through modulating epithelial-mesenchymal transition by targeting Snail1. Toxicol Lett. 358:17-26.
11. Mazumdar R, Eberhart JK. 2023 Loss of nicotinamide nucleotide transhydrogenase sensitizes embryos to ethanol-induced neural crest and neural apoptosis via generation of reactive oxygen species. Front Neurosci. 17:1154621.
12. Burton DF, Boa-Amponsem OM, Dixon MS, Hopkins MJ, Herbin TA, Toney S, Tarpley M, Rodriguez BV, Fish EW, Parnell SE, Cole GJ, Williams KP. 2022. Pharmacological activation of the Sonic hedgehog pathway with a Smoothened small molecule agonist ameliorates the severity of alcohol-induced morphological and behavioral birth defects in a zebrafish model of fetal alcohol spectrum disorder. J Neurosci Res.100(8):1585-1601.
13. Petrelli B, Bendelac L, Hicks GG, Fainsod A. 2019. Insights into retinoic acid deficiency and the induction of craniofacial malformations and microcephaly in fetal alcohol spectrum disorder. Genesis. 57:e23278.
14. Chen X, Liu J, Chen SY. 2013. Over-expression of Nrf2 diminishes ethanol-induced oxidative stress and apoptosis in neural crest cells by inducing an antioxidant response. Repro Tox. 42:102-109.
15. Flentke GR, Garic A, Amberger E, Hernandez M, Smith SM. 2011. The calcium-

mediated repression of β-catenin and its transcriptional signaling mediates neural crest cell death in an avian model of fetal Alcohol Syndrome. Birth Defects Res A. 91:591-602.
16. Fish EW, Tucker SK, Peterson RL, Eberhart JK, Parnell SE. 2021. Loss of tumor protein 53 protects against alcohol-induced facial malformations in mice and zebrafish. Alcohol Clin Exp Res. 45(10):1965-1979.
17. Flentke GR, Baulch JW, Berres ME, Garic A, Smith SM. 2019. Alcohol-mediated calcium signals dysregulate pro-survival Snai2/PUMA/Bcl2 networks to promote p53-mediated apoptosis in avian neural crest progenitors. Birth Defects Res. 111:686-699.
18. Berres ME, Garic A, Flentke GR, Smith SM. 2017. Transcriptome profiling identifies ribosome biogenesis as a target of alcohol teratogenicity and vulnerability during early embryogenesis. PLoS ONE e0169351.
19. Li F, Lin J, Li T, Jian J, Zhang Q, Zhang Y, Liu X, Li Q. 2022. Rrn3 gene knockout affects ethanol-induced locomotion in adult heterozygous zebrafish. Psychopharmacology (Berl) 239(2):621-630.
20. Green ML, Singh AV, Zhang Y, Nemeth KA, Sulik KK, Knudsen TB. 2007. Reprogramming of genetic networks during initiation of the Fetal Alcohol Syndrome. Dev Dyn. 236:613-631.
21. Downing C, Balderrama-Durbin C, Kimball A, Biers J, Wright H, Gilliam D, Johnson TE. 2012. Quantitative trait locus mapping for ethanol teratogenesis in BXD recombinant inbred mice. Alcohol Clin Exp Res. 36:1340-1354.
22. Garic A, Berres ME, Smith SM. 2014. High-throughput transcriptome sequencing identifies candidate genetic modifiers of vulnerability to fetal alcohol spectrum disorders. Alcohol Clin Exp Res. 38:1874-1882.
23. Huang Y, Flentke GR, Rivera OC, Saini N, Mooney SM, Smith SM. 2024. Alcohol exposure induces nucleolar stress and apoptosis in mouse neural stem cells and late-term fetal brain. Cells 13(5):440.
24. Flentke GR, Wilkie TE, Baulch J, Huang Y, Smith SM. 2024. Alcohol exposure suppresses ribosome biogenesis and causes nucleolar stress in cranial neural crest cells.. PLoS One. 19(6):e0304557.
25. Warner JR, Vilardell J, Sohn JH. 2001. Economics of ribosome biosynthesis. Cold Spr Harbor Symp Quant Biol LXVI:567-574.
26. Nomura M. 2001. Ribosomal RNA genes, RNA polymerases, nucleolar structures, and synthesis of rRNA in the yeast Saccharomyces cerevisiae. Cold Spring Harb Symp Quant Biol 66:555-565.
27. Dibble CC, Manning BD. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. Nature Cell Biol. 15:555-564.
28. Chauvin C, Koka V, Nouschi A, Mieulet V, Hoareau-Aveilla C, Dreazen A, Cagnard N, Carpentier W, Kiss T, Meyuhas O, Pende M. 2014. Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. Oncogene. 33:474-83.
29. Boulon S, Westman BJ, Hutten S, Boisvert F-M, Lamond AI. 2010. The nucleolus under stress. Mol Cell 40:216-227.
30. Lafita-Navarro MC, Conacci-Sorrell M. 2023. Nucleolar stress: From development to cancer. Semin Cell Dev Biol. 136:64-74.
31. Chakraborty A, Uechi T, Kenmochi N. 2011. Guarding the 'translation apparatus': defective ribosome biogenesis and the p53 signaling pathway. WIREs RNA 2:507-522.
32. Deisenroth C, Franklin DA, Zhang Y. 2016. The evolution of the ribosomal protein-

MDM2-p53 pathway. Cold Spring Harb Perspect Med. 6(12):a026138.
33. Daftuar L, Zhu Y, Jacq X, Prives C. 2013. Ribosomal proteins RPL37, RPS15, and RPS20 regulate the Mdm2-p53-MdmX network. PLoS ONE 8:e68667.
34. Yelick PC, Trainor PA. 2015. Ribosomopathies: global process, tissue specific defects. Rare Dis 3(1):e1025185
35. Trainor PA. 2014. Craniofacial birth defects: the role of neural crest cells in the etiology and pathogenesis of Treacher Collins syndrome and the potential for prevention. Am J Med Genet Part A 152A:2984-2994.
36. Kang J, Brajanovski N, Chan KT, Xuan J, Pearson RB, Sanij E. 2021. Ribosomal proteins and human diseases: molecular mechanisms and targeted therapy. Signal Transduct Target Ther. 6:323.
37. Ishii M, Arias AC, Liu L, Chen YP, Bronner ME, Maxson RE. 2012. A stable cranial neural crest cell line from mouse. Stem Cells Dev. 21:3069-80.
38. Nguyen BH, Ishii M, Maxson RE, Wang J. 2018. Culturing and manipulation of O9-1 neural crest cells. J Vis Exp. 140:58346.
39. Wang J, Xiao Y, Hsu CW, Martinez-Traverso IM, Zhang M, Bai Y, Ishii M, Maxson RE, Olson EN, Dickinson ME, Wythe JD, Martin JF. 2016. Yap and Taz play a crucial role in neural crest-derived craniofacial development. Development. 143:504-515.
40. Pascual F, Icyuz M, Karmaus P, Brooks A, Van Gorder E, Fessler MB, Shaw ND. 2023. Cholesterol biosynthesis modulates differentiation in murine cranial neural crest cells. Sci Rep. 13:7073.
41. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley JL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines - minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611-622.
42. Lee-Fruman KK, Kuo CJ, Lippincott J, Terada N, Blenis J. 1999. Characterization of S6K2, a novel kinase homologous to S6K1. Oncogene. 18:5108-5114.
43. Steinberg GR, Hardie DG. 2023. New insights into activation and function of the AMPK. Nat Rev Mol Cell Biol. 24:255-272.
44. Trefts E, Shaw RJ. 2021. AMPK: restoring metabolic homeostasis over space and time. Mol Cell. 81:3677-3690.
45. Romanyuk N, Sintakova K, Arzhanov I, Horak M, Gandhi C, Jhanwar-Uniyal M, Jendelova P. 2024. mTOR pathway inhibition alters proliferation as well as differentiation of neural stem cells. Front Cell Neurosci 18:1298182.
46. McCarthy N, Wetherill L, Lovely CB, Swartz ME, Foroud TM, Eberhart JK. 2013. Pdgfra protects against ethanol-induced craniofacial defects in a zebrafish model of FASD. Development. 140:3254-3265.
47. Li Q, Ren J. 2007. Chronic alcohol consumption alters mammalian target of rapamycin (mTOR), reduces ribosomal p70S6 kinase and p4E-BP1 levels in mouse cerebral cortex. Exp Neurol 204:840-844.
48. Lee J, Lunde-Young R, Naik V, Ramirez J, Orzabal M, Ramadoss J. 2020. Chronic binge alcohol exposure during pregnancy alters mTOR system in rat fetal hippocampus. Alcohol Clin Exp Res. 44:1329-1336.
49. Luo J. Autophagy and ethanol neurotoxicity. 2014. Autophagy. 10:2099-2108.
50. Chen G, Ke Z, Xu M, Liao M, Wang X, Qi Y, Zhang T, Frank JA, Bower KA, Shi X, Luo J. 2012. Autophagy is a protective response to ethanol neurotoxicity. Autophagy. 8:1577-1589.
51. Sangaunchom P, Dharmasaroja P. 2020. Caffeine potentiates ethanol-induced

neurotoxicity through mTOR/p70S6K/4E-BP1 inhibition in SH-SY5Y cells. Int J Toxicol 39:131-140.
52. Hong-Brown LQ, Brown CR, Kazi AA, Navaratnarajah M, Lang CH. 2012. Rag GTPases and AMPK/TSC2/Rheb mediate the differential regulation of mTORC1 signaling in response to alcohol and leucine. Am J Physiol Cell Physiol 302:C1557-C1565.
53. Nie X, Zheng J, Ricupero CL, He L, Jiao K, Mao JJ. 2018. mTOR acts as a pivotal signaling hub for neural crest cells during craniofacial development. PLoS Genet 14:e1007491.
54. Xie M, Kaiser M, Gershtein Y, Schnyder D, Deviatiiarov R, Gazizova G, Shagimardanova E, Zikmund T, Kerckhofs G, Ivashkin E. 2024. The level of protein in the maternal murine diet modulates the facial appearance of the offspring via mTORC1 signaling. Nat Comm 15:1-15.
55. Burstyn-Cohen T, Kalcheim C. 2002. Association between the cell cycle and neural crest delamination through specific regulation of G1/S transition. Dev Cell 3:383-395.
56. Ridenour DA, McLennan R, Teddy JM, Semerad CL, Haug JS, Kulesa PM. 2014. The neural crest cell cycle is related to phases of migration in the head. Development. 141:1095-103.
57. Dwyer DS, Bradley RJ. 2000. Chemical properties of alcohols and their protein binding sites. Cell Mol Leif Sci. 57:265-275.
58. Garic-Stankovic A, Hernandez M, Flentke GR, Smith SM. 2006. Structural constraints for alcohol-stimulated Ca2+ release in neural crest, and dual agonist/antagonist properties of n-octanol. Alcohol Clin Exp Res 30:552-559.
59. Garic-Stankovic A., Hernandez MA, Flentke GR, Debelak-Kragtorp KA, Armant DR, Smith SM. 2005. Ethanol triggers neural crest apoptosis thru the selective activation of a pertussis toxin-sensitive G-protein and a phospholipase Cβ-dependent Ca2+ transient. Alcohol Clin Exp Res 29:1237-1246.
60. Flentke GR, Klingler RH, Tanguay RL, Carvan MJ3rd, Smith SM. 2014. An evolutionarily-conserved mechanism of calcium-dependent neurotoxicity in a zebrafish model of FASD. Alcohol Clin Exp Res. 38:1255-1265.
61. Srivastava VK, Hiney JK, Dees WL. 2018. Alcohol delays the onset of puberty in the female rat by altering key hypothalamic events. Alcohol Clin Exp Res 42:1166-1176.
62. Xu S, Jeong SJ, Li G, Koo JW, Kang UG. 2020. Repeated ethanol exposure influences key enzymes in cholesterol and lipid homeostasis via the AMPK pathway in the rat prefrontal cortex. Alcohol 85:49-56.
63. Hong-Brown LQ, Brown CR, Kazi AA, Huber DS, Pruznak AM, Lang CH. 2010. Alcohol and PRAS40 knockdown decrease mTOR activity and protein synthesis via AMPK signaling and changes in mTORC1 interaction. J Cell Biochem 109:1172-1184.
64. Hong-Brown LQ, Brown CR, Kazi AA, Navaratnarajah M, Lang CH. 2012. Rag GTPases and AMPK/TSC2/Rheb mediate the differential regulation of mTORC1 signaling in response to alcohol and leucine. Am J Physiol Cell Physiol 302:C1557-C1565.
65. Kandadi MR, Hu N, Ren J. 2013. ULK1 plays a critical role in AMPK-mediated myocardial autophagy and contractile dysfunction following acute alcohol challenge. Curr Pharm Des 19:4874-4887.
66. Yuan F, Xu Y, You K, Zhang J, Yang F, Li YX. 2021. Calcitriol alleviates ethanol-induced hepatotoxicity via AMPK/mTOR-mediated autophagy. Arch Biochem Biophys 697:108694.
67. Eom JW, Lee JM, Koh JY, Kim YH. 2016. AMP-activated protein kinase contributes to zinc-induced neuronal death via activation by LKB1 and induction of Bim in mouse cortical cultures. Mol Brain 9:14.

68. Xu Y, Liu C, Chen S, Ye Y, Guo M, Ren Q, Liu L, Zhang H, Xu C, Zhou Q, Huang S, Chen L. 2014. Activation of AMPK and inactivation of Akt result in suppression of mTOR-mediated S6K1 and 4E-BP1 pathways leading to neuronal cell death in in vitro models of Parkinson's disease. Cell Signal 26:1680-1689.
69. Marcelo KL, Means AR, York B. 2016. The Ca2+/calmodulin/CaMKK2 axis: nature's metabolic CaMshaft. Trends Endocrin Metab. 27:706-718.
70. Naseer M, Ullah I, Narasimhan M, Lee H, Bressan R, Yoon G, Yun D, Kim M. 2014. Neuroprotective effect of osmotin against ethanol-induced apoptotic neurodegeneration in the developing rat brain. Cell Death Dis 5:e1150-e1150.

Figure Legends

Figure 1. Alcohol rapidly represses ribosome biogenesis and causes p53-mediated apoptosis in primary neural crest cells. (A) ITS-1, a product of de novo rRNA synthesis, is reduced by 1hr following exposure to alcohol. **(B)** Nucleoli in ALC neural crest (4hr thereafter) are reduced and become diffused, as visualized using immunostain for the nucleolar protein UBF. **(C)** p53 is stabilized in ALC neural crest within 2hr of exposure and this persists for at least 12hr. **(D)** Alcohol causes apoptosis in neural crest at 16hr post-exposure. Red signal is TUNEL, blue is DAPI stain to visualize nuclei. All studies use 80mM alcohol. Values are mean ± SD with N=3 independent replicates. * $p<0.05$, ** $p<0.001$ compared with CON by either two-tailed T-test (D) or one-way ANOVA (A,C).

Figure 2. Alcohol reduces the positive effector of ribosome biogenesis p70/S6K. (A) Western blot analysis for phospho- and total p70/S6K at the indicated times following alcohol exposure. Total p70/S6K is reduced by 2hr post-exposure; the ratio of p-p70/S6K to p70/S6K is reduced by 12hr as compared with control (CON). **(B-E)** Overexpression of gain-of-function p70/S6K **(B)** enhances its protein abundance in ALC but not CON neural crest, **(C)** normalizes rRNA synthesis in ALC neural crest and reduces it in CON, **(D)** prevents the elevated p53 content in ALC neural crest, and **(E)** prevents alcohol-induced apoptosis, as assessed by TUNEL. All studies use 80mM alcohol. Values are mean ± SD with N=3 independent replicates, and analyzed using one-way ANOVA compared with CON.

Figure 3. Alcohol represses the upstream p70/S6K effector TORC1in neural crest. (A) Alcohol increases the content of p-mTOR (S2446/2448) by 2hr following exposure and this

elevation persists for at least 12hr. **(B)** Alcohol increases the active phospho-forms of the TORC1 upstream negative effectors p-TSC2(S1387) and pRaptor(S72) within 2hr to 4hr of alcohol exposure. **(C)** Alcohol reduces the downstream TORC1 target p-4E-BP1. **(D)** Alcohol does not affect the abundance of the TORC2 effectors pRictor, Deptor, or PKCα. Values are mean ± SD with N=3 independent replicates and analyzed using one-way ANOVA compared with CON.

Figure 4. Treatment with the TORC1 inhibitor rapamycin or the TORC1 activator L-leucine respectively activates or prevents alcohol's impact on rRNA synthesis, p53 stability, and apoptosis in neural crest. (A-C) Rapamycin studies. **(A)** Rapamycin reduces rRNA synthesis in ALC but not CON neural crest at 2hr post-exposure. **(B)** Rapamycin stabilizes p53 protein in CON and ALC neural crest. **(C)** Rapamycin causes apoptosis in ALC but not CON neural crest. Values are mean ± SD with N=3 independent replicates and analyzed using one-way ANOVA compared with CON. **(D-G)** L-Leucine studies. **(D)** A 2hr pretreatment with L-leucine enhances rRNA synthesis in CON and normalizes it in ALC neural crest. **(E)** L-leucine further increases pAMPK and normalizes total p70/S6K, but not p-p70/S6K in ALC neural crest. **(F)** L-leucine reduces p53 protein content in ALC neural crest. **(G)** L-Leucine normalizes apoptosis in ALC neural crest. For all, values are mean ± SD with N=3 independent replicates and analyzed using one-way ANOVA compared with CON.

Figure 5. Alcohol rapidly elevates pAMPK in neural crest, and the AMPK inhibitor dorsomorphin counters alcohol's effects on AMPK-TORC1 signaling, rRNA synthesis, and p53-mediated apoptosis. (A) Alcohol exposure increases pAMPK within 2hr of exposure and it remains elevated for at least 18hr. **(B)** The AMPK inhibitor dorsomorphin normalizes pAMPK,

pRaptor, p-p70/S6K, and p-mTOR in ALC neural crest. **(C-E)** Dorsomorphin also normalizes rRNA synthesis **(C)**, p53 protein content **(D)**, and apoptosis **(E)** in ALC neural crest. Values are mean ± SD with N=3 independent replicates and analyzed using one-way ANOVA compared with CON.

Figure 6. The AMPK activator AICAR further alters AMPK and p70/S6K, suppresses rRNA synthesis and exacerbates p53-mediated apoptosis in ALC neural crest, but not in CON. (A) AICAR further exacerbates pAMPK activation in ALC neural crest at 2hr post-exposure, but not in CON. **(B)** AICAR further reduces p-p70/S6K in ALC neural crest. **(C-E)** AICAR further impacts rRNA synthesis **(C)** p53 protein content, **(D)** and apoptosis **(E)** in ALC neural crest, but is insufficient to affect these in CON. Values are mean ± SD with N=3 independent replicates and analyzed using one-way ANOVA compared with CON.

Figure 7. Diagram of alcohol's proposed mechanism of action in mediating neural crest apoptosis. Data herein and in [24] suggest that alcohol's activation of pAMPK activates the TORC1 repressors pTSC2 and pRaptor to reduces TORC1's stimulation of ribosome biogenesis via p70/S6K, resulting in nucleolar stress and the activation of p53/MDM2-mediated apoptosis.

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

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Figures 1-7

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

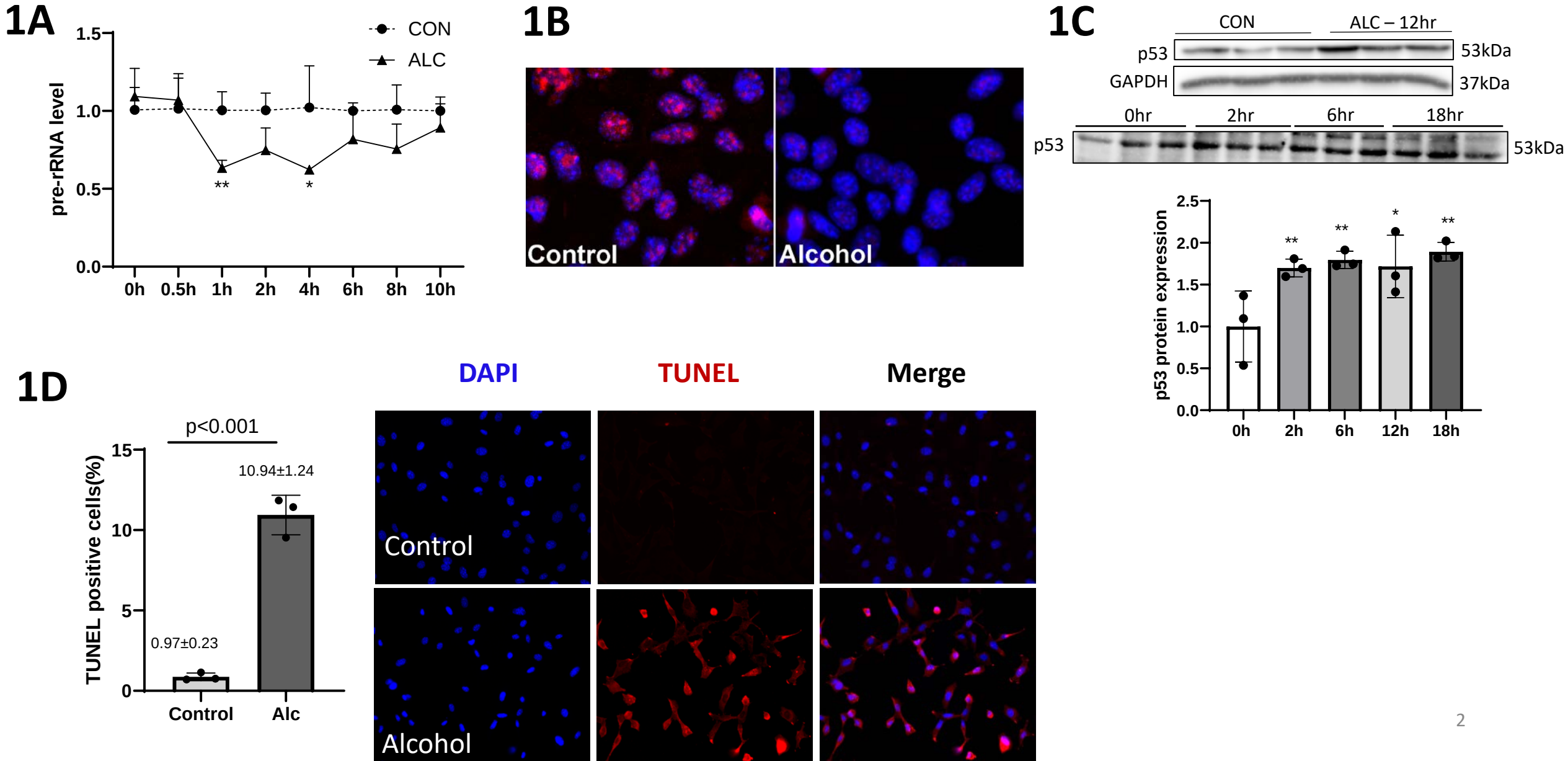


Figure 2A. Alcohol exposure reduces the RBG effector p70/S6K1 in NC.

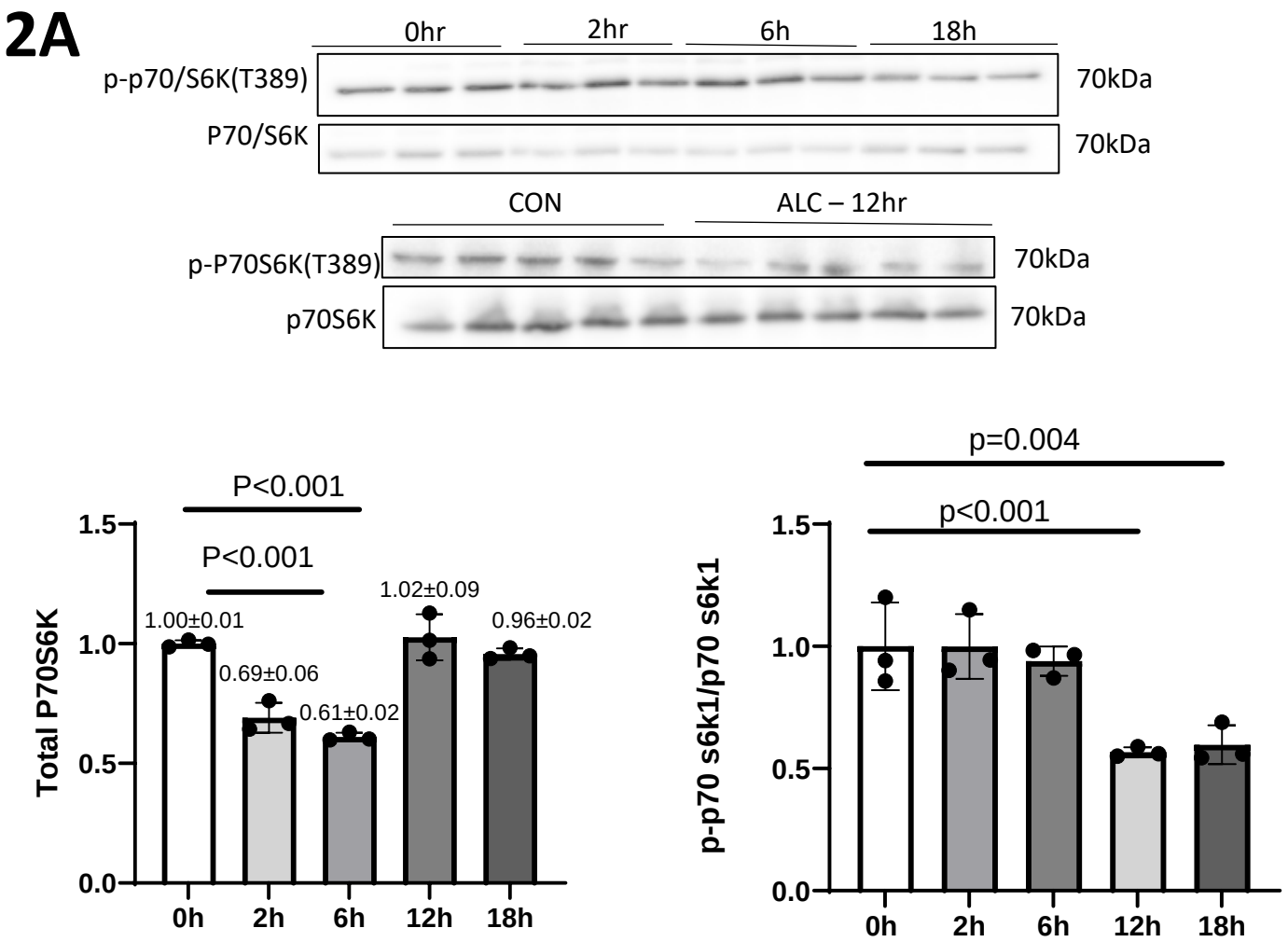
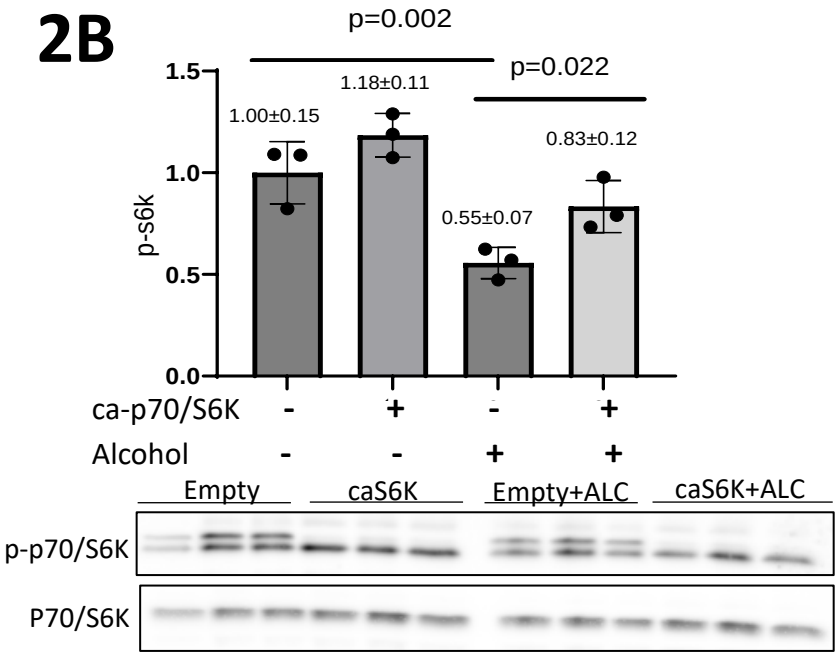
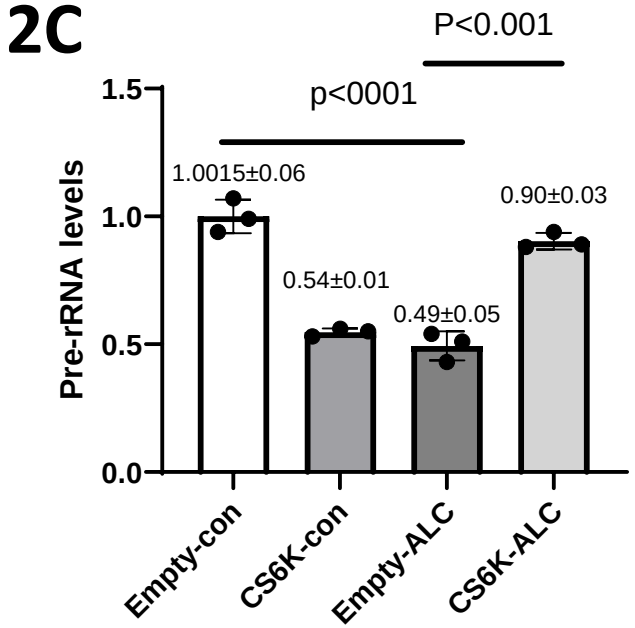
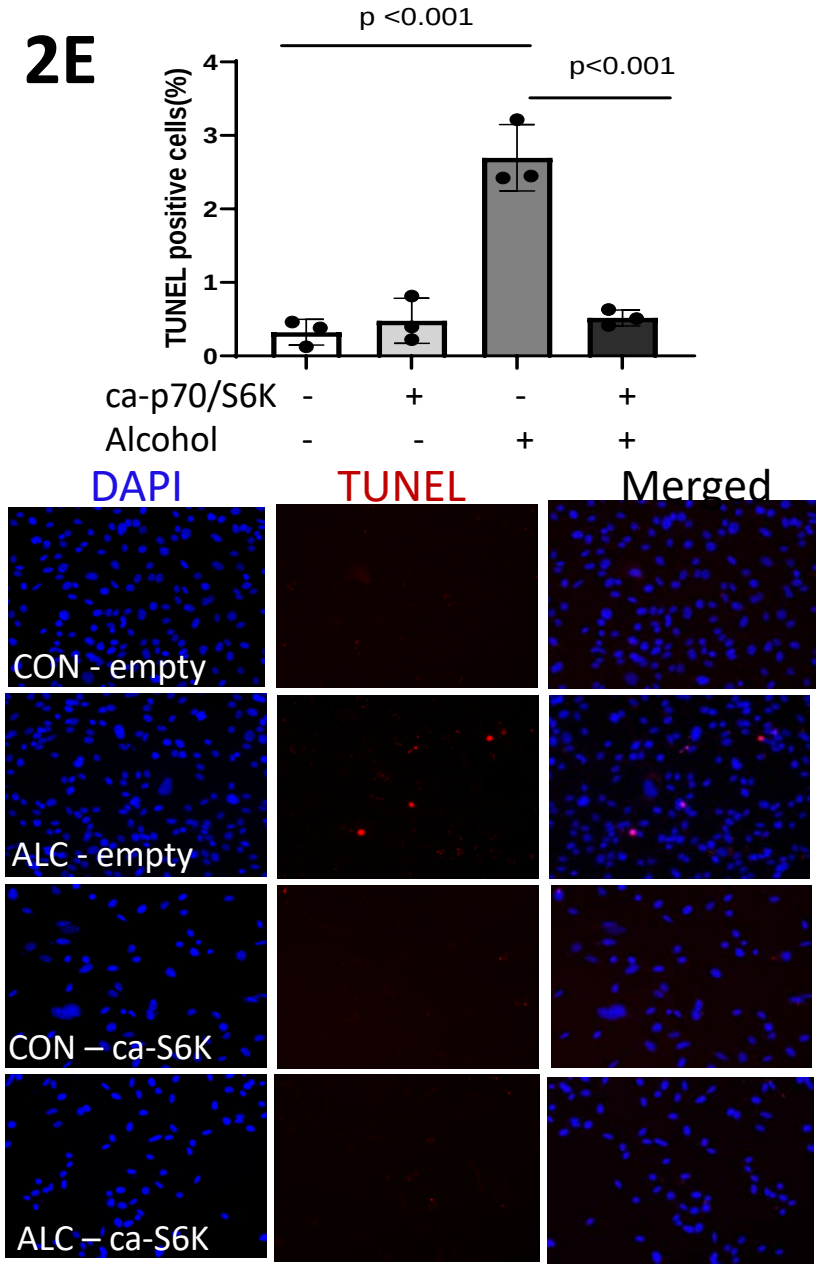
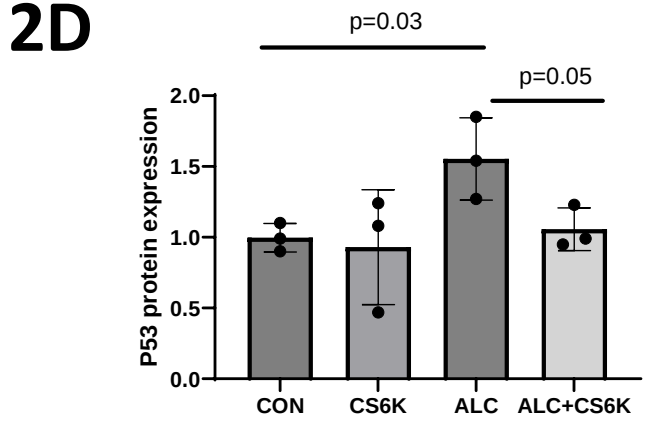


Figure 2B-E. Gain-of-function p70/S6K normalizes S6K & p53, and prevents the loss of rRNA synthesis and apoptosis in alcohol-exposed NC.









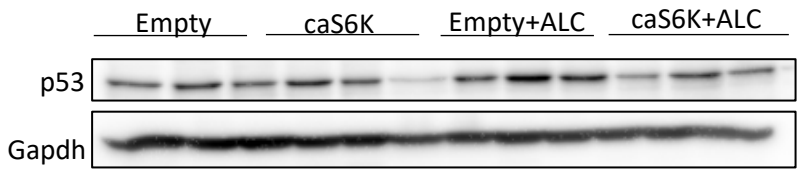


Fig. 3A, B. Alcohol increases phosphorylation of the p70/S6K effector mTOR (S2446/2448) and the negative mTOR effectors TSC2(S1387) & pRaptor(S72)

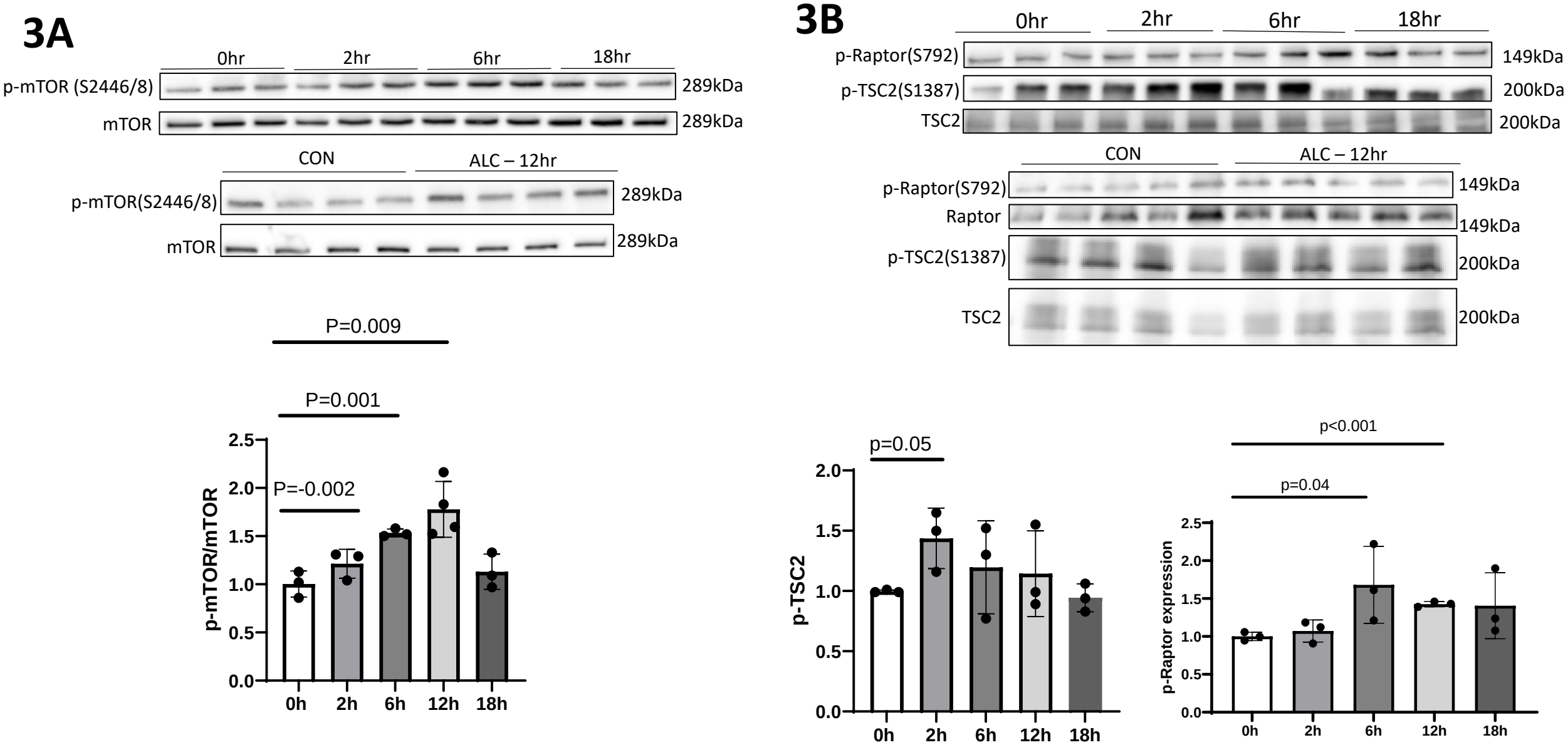
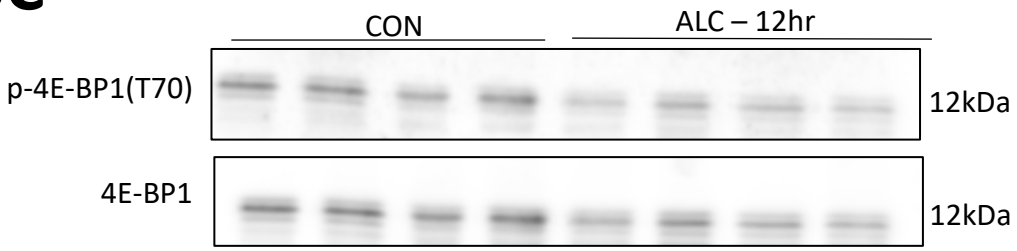
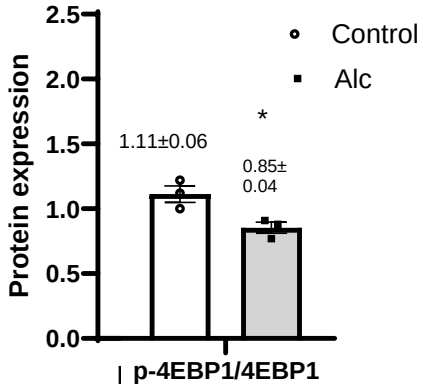


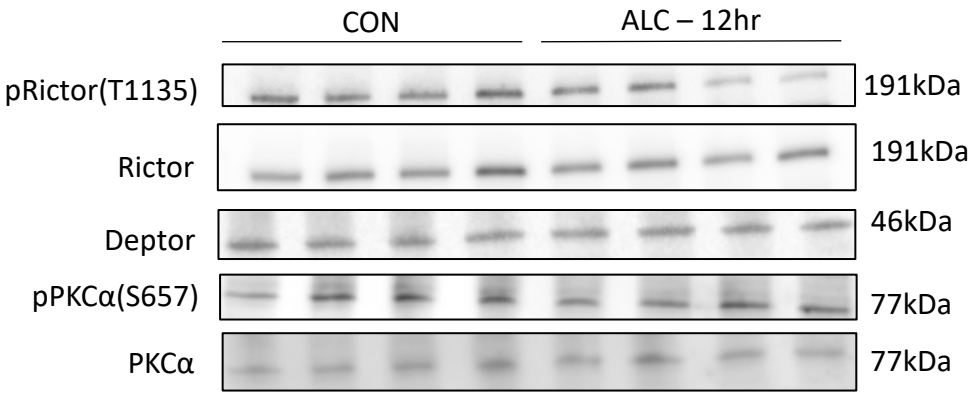
Figure 3C,D. ALC reduces the downstream TORC1 target p4E-BP1 but does not alter the abundance of TORC2 effectors pRictor, Deptor, or pPKCα.

3C





3D



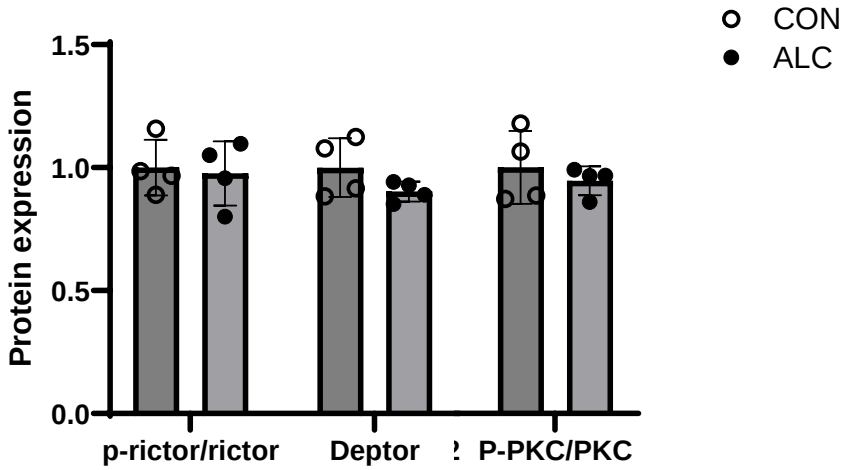


Figure 4A-C. The TORC1 antagonist Rapamycin exacerbates alcohol-induced apoptosis but is not sufficient to suppress rRNA synthesis and enhanced p53 stability and apoptosis.

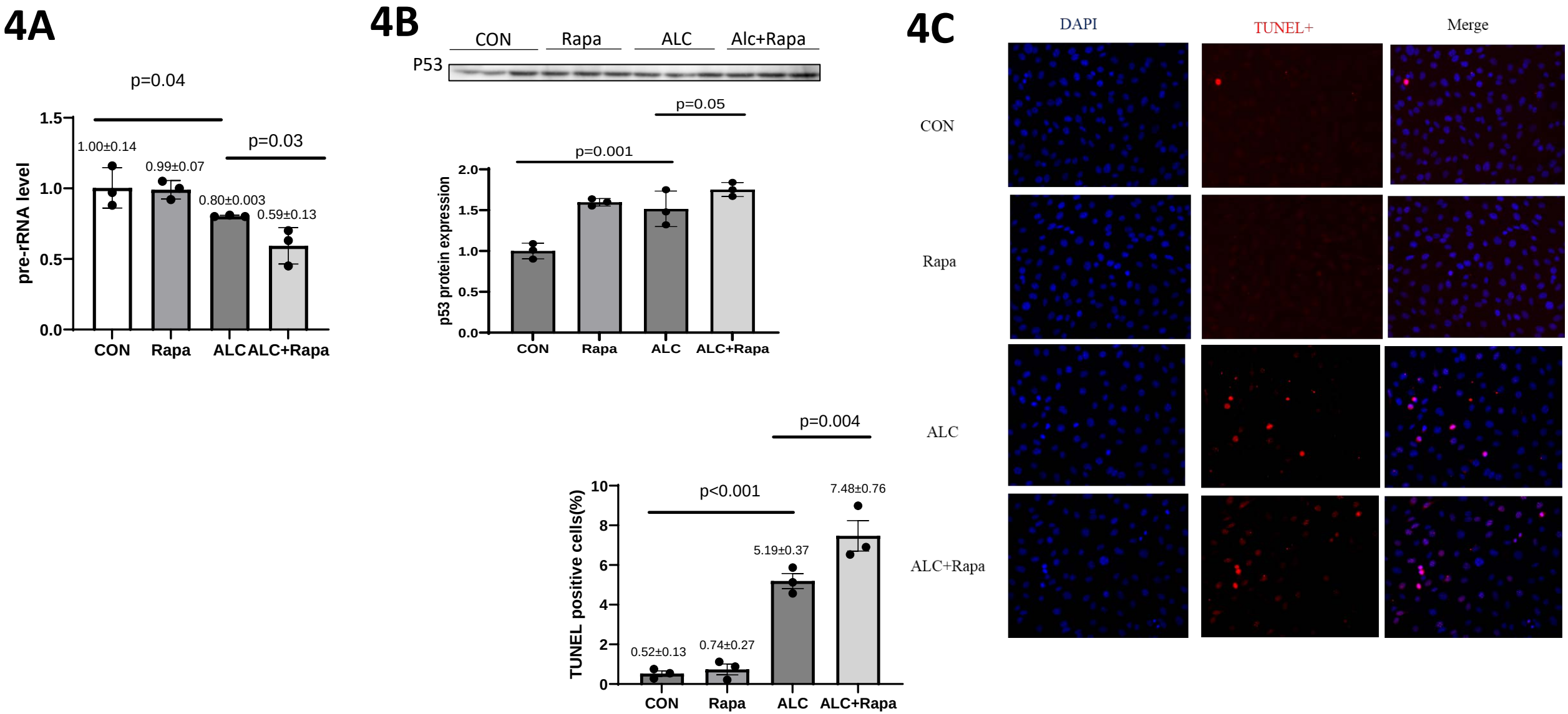
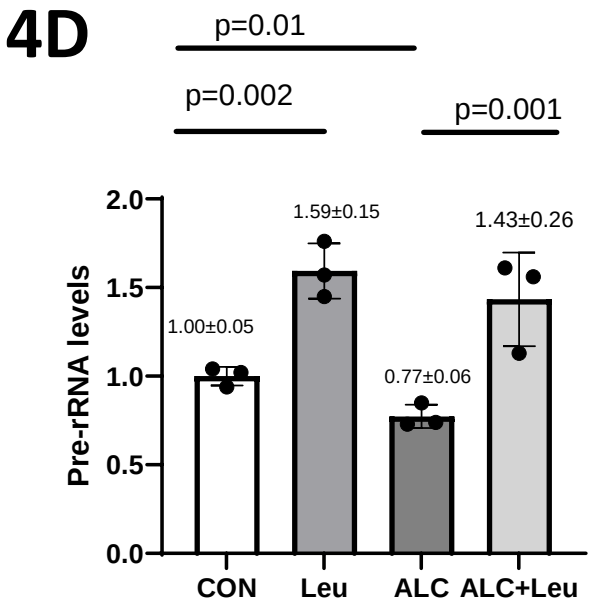
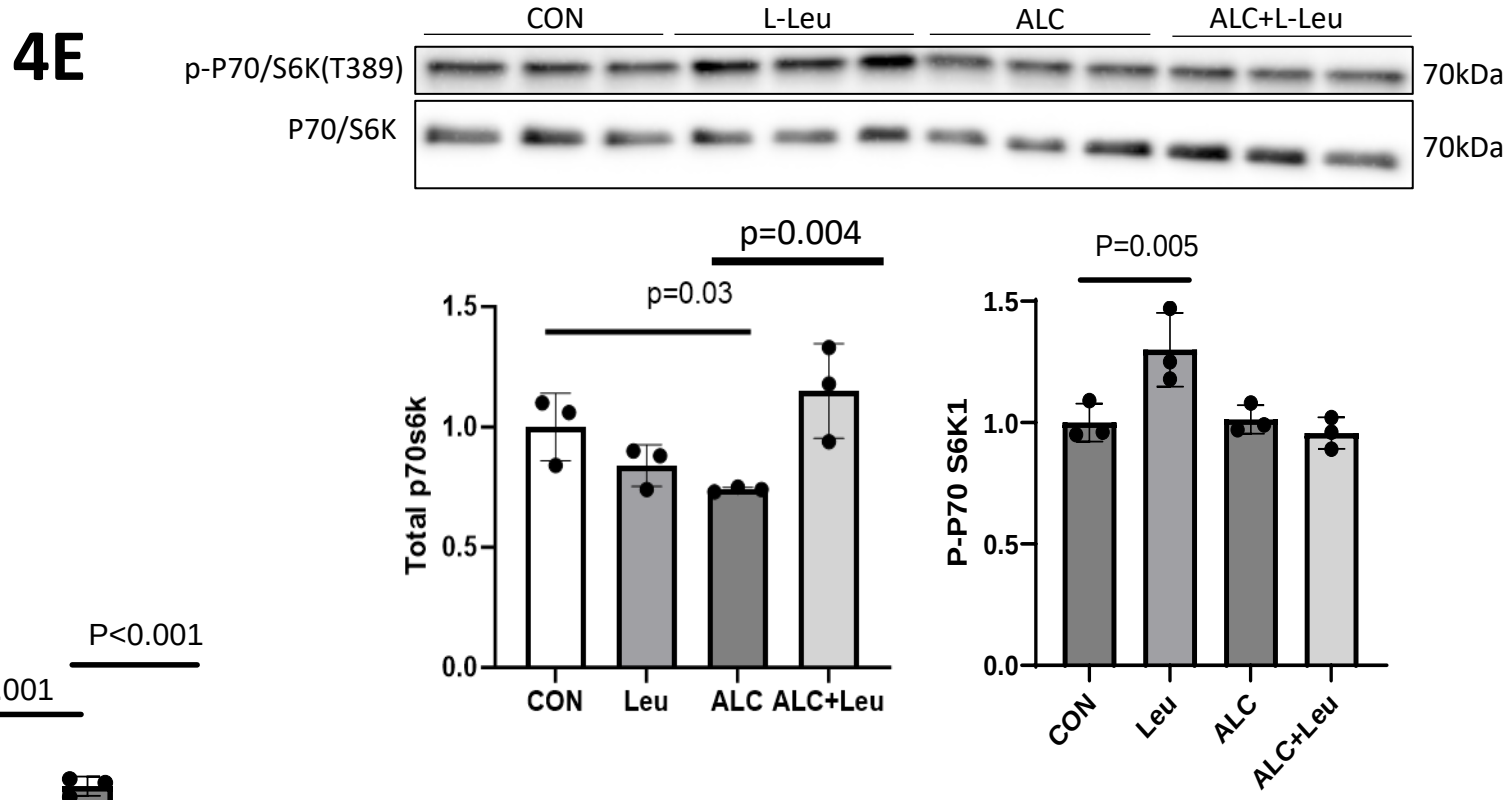


Figure 4D-F. The TORC1 activator L-Leucine normalizes rRNA synthesis, total p70/S6K, and p53 content in ALC neural crest.





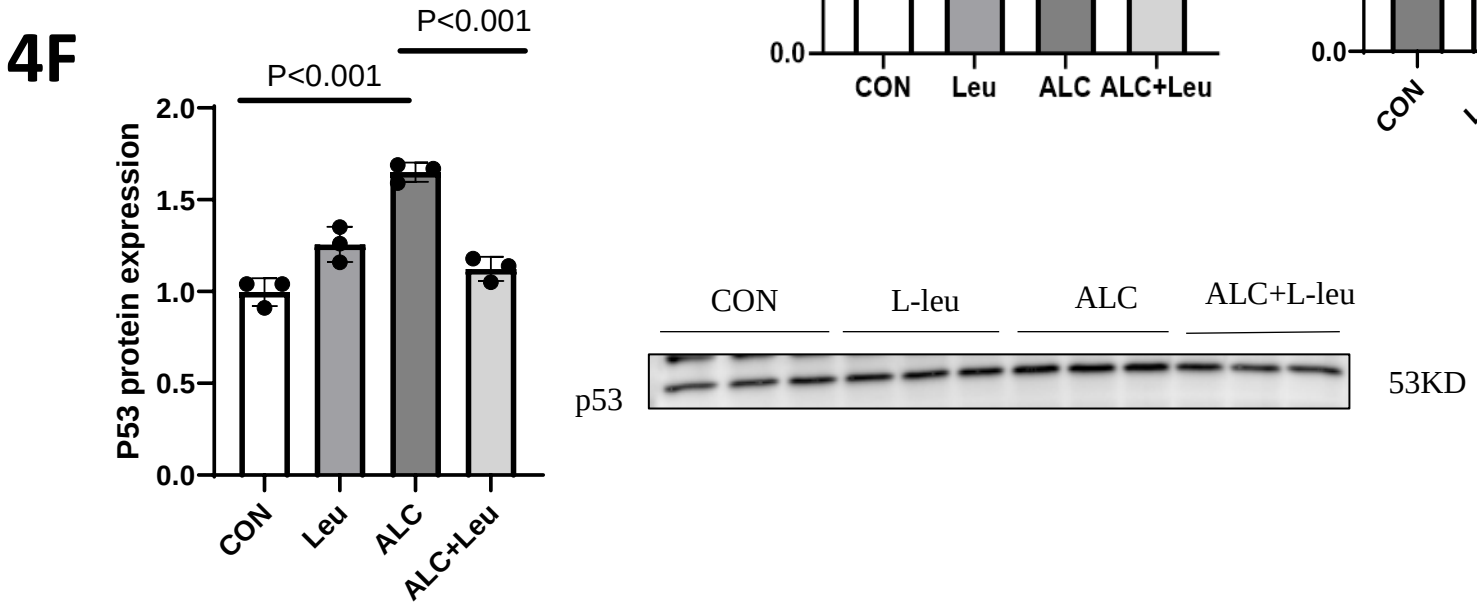
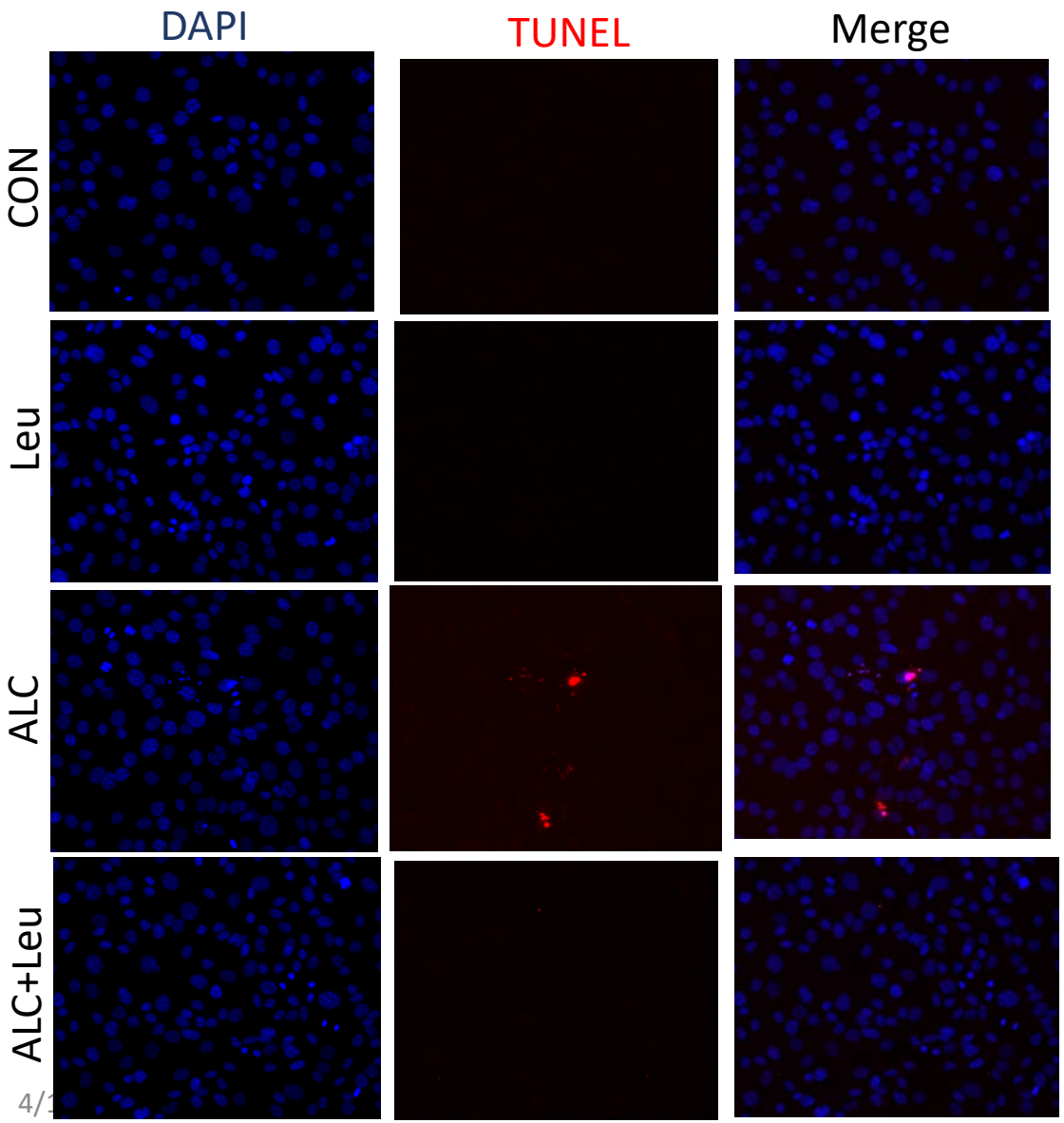


Figure 4G. The TORC1 activator L-Leucine normalizes apoptosis in ALC neural crest.

4G



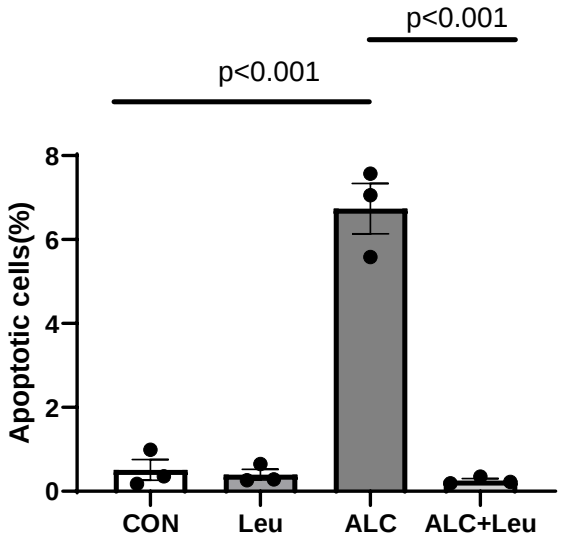
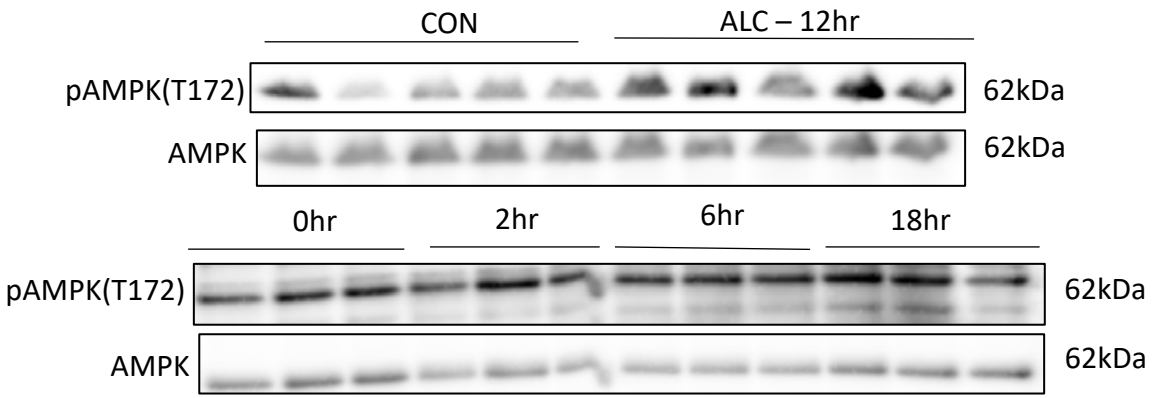
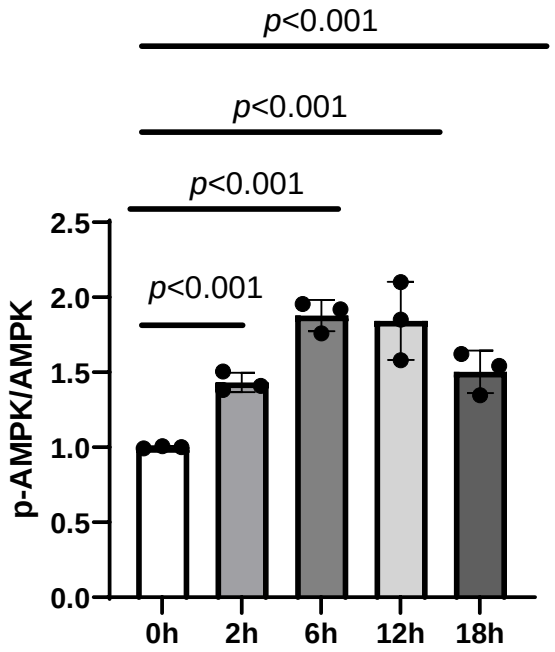


Fig. 5A. Alcohol exposure rapidly elevates pAMPK in neural crest.

5A





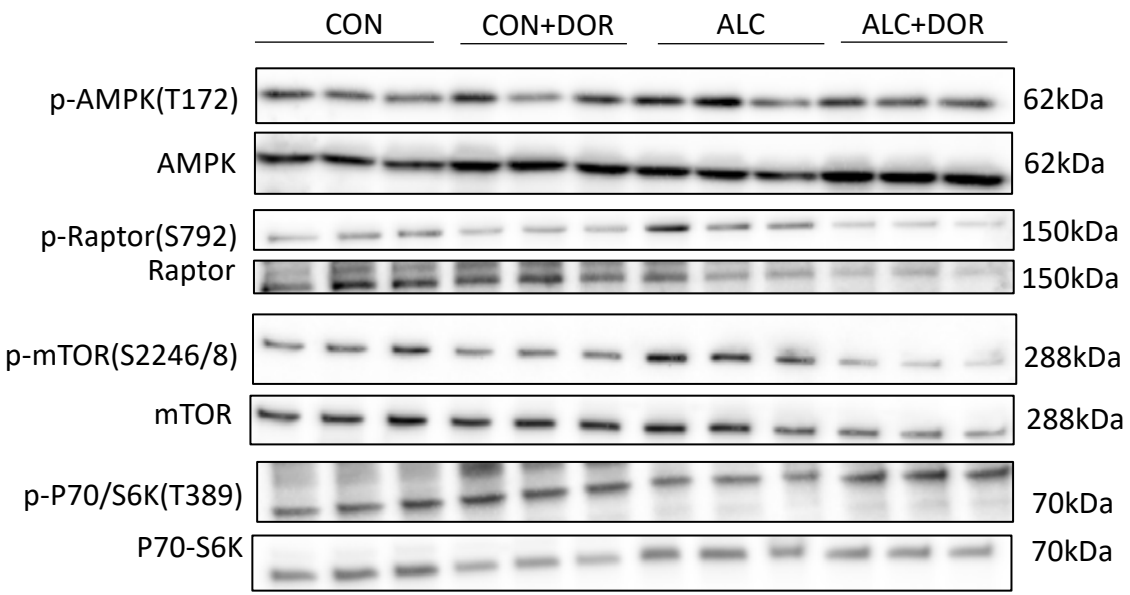


Fig 5B. The AMPK inhibitor Dorsomorphin reduces pAMPK & pRaptor, and increases p-mTOR and p-p70/S6K in ALC neural crest.

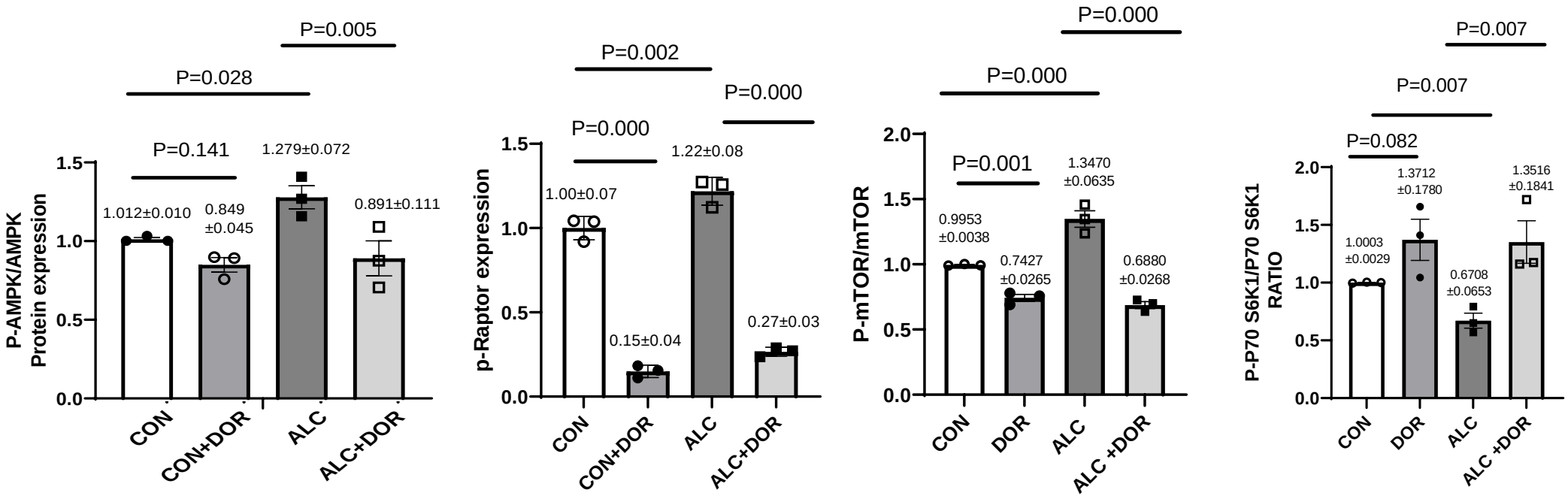
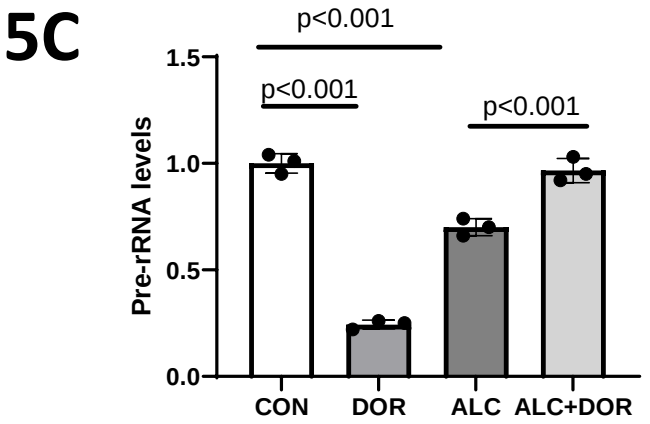
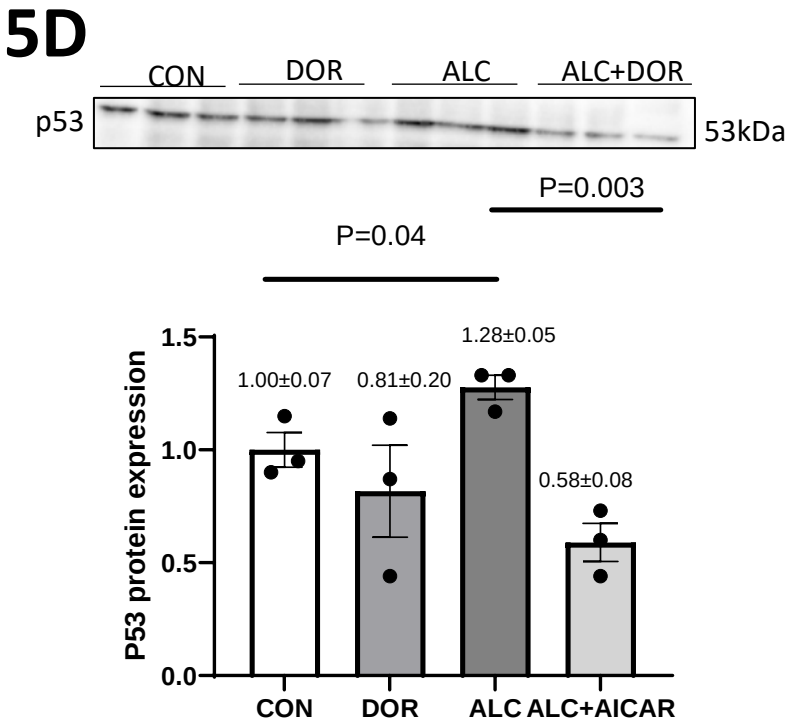
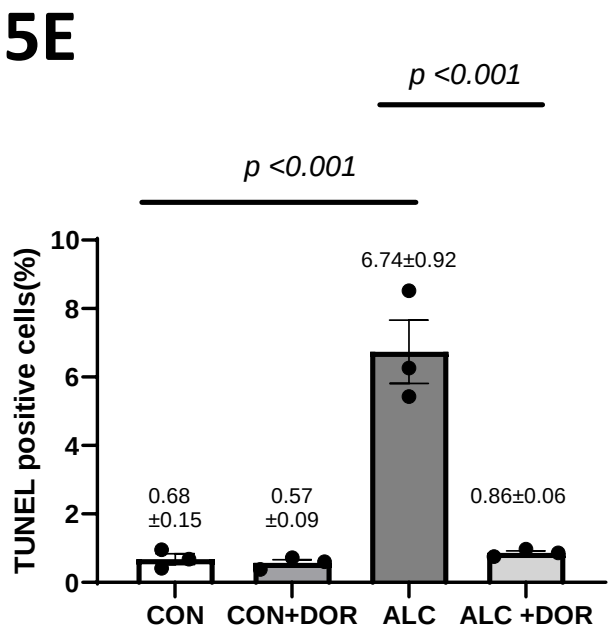


Figure 5C-E. The AMPK inhibitor Dorsomorphin prevents ALC-mediated loss of rRNA synthesis, p53 stabilization, and apoptosis in neural crest.







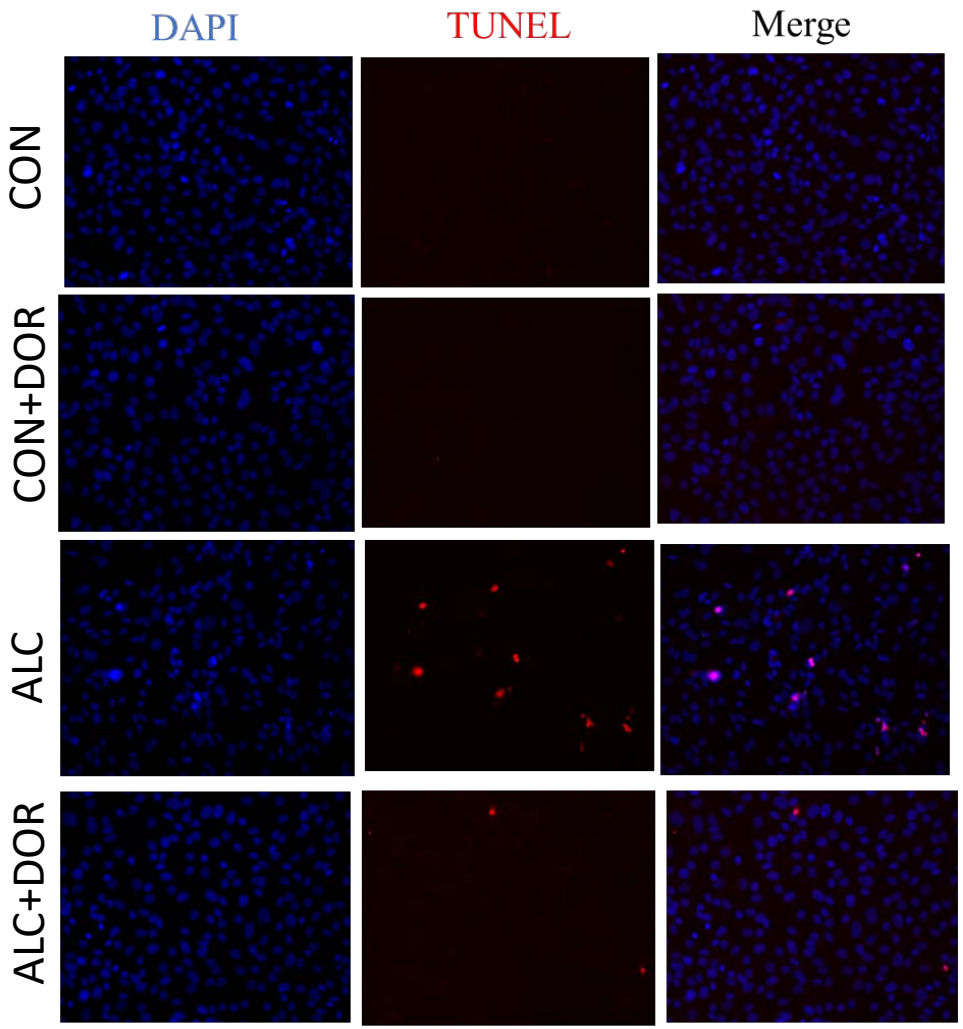
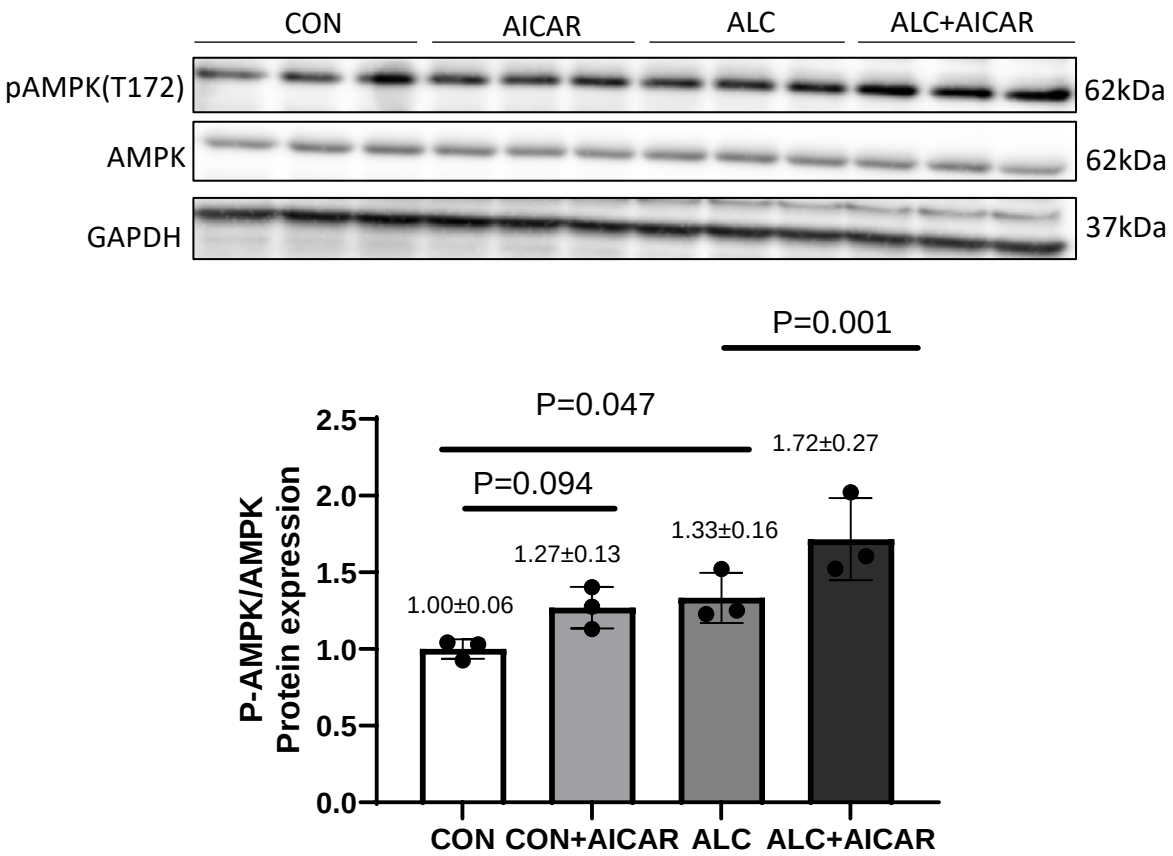


Fig. 6A,B. The AMPK activator AICAR exacerbates ALC-induced pAMPK and p-p70/S6K reductions, and increases pAMPK but does not reduce p-p70/S6K in normal neural crest.

6A



6B

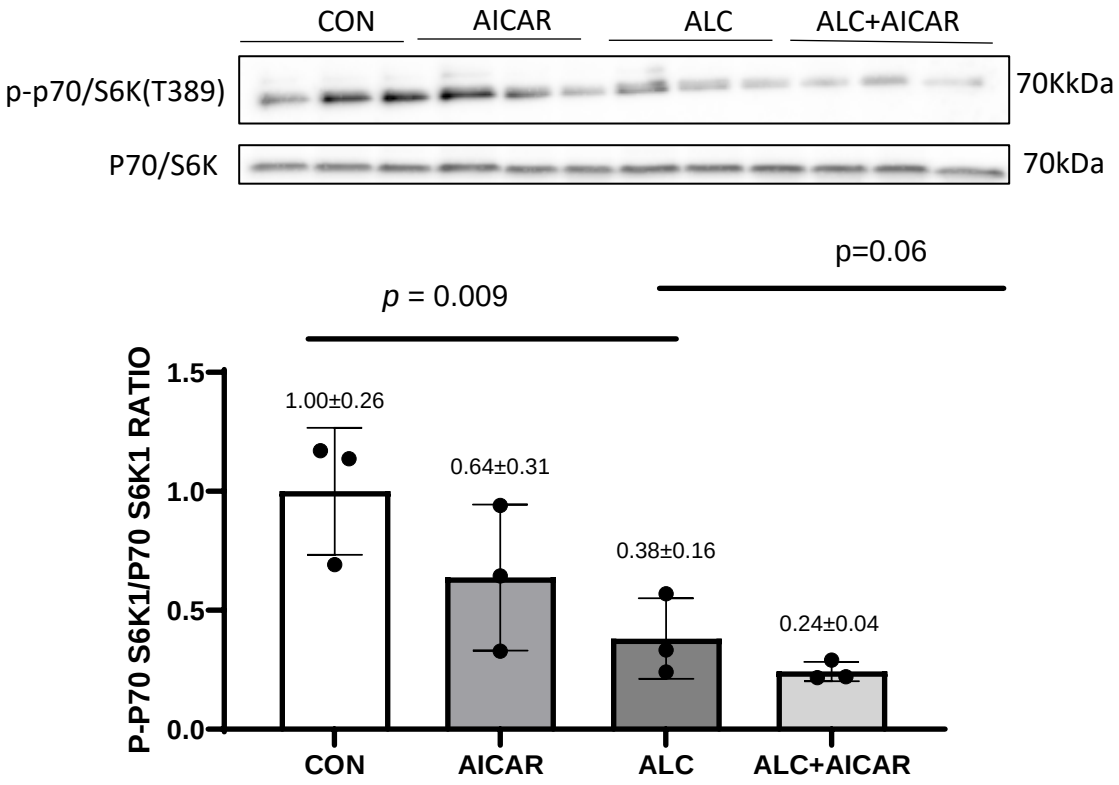
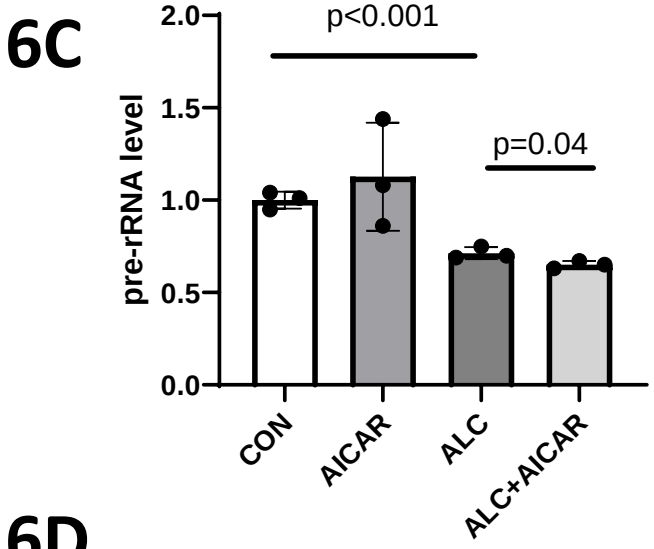
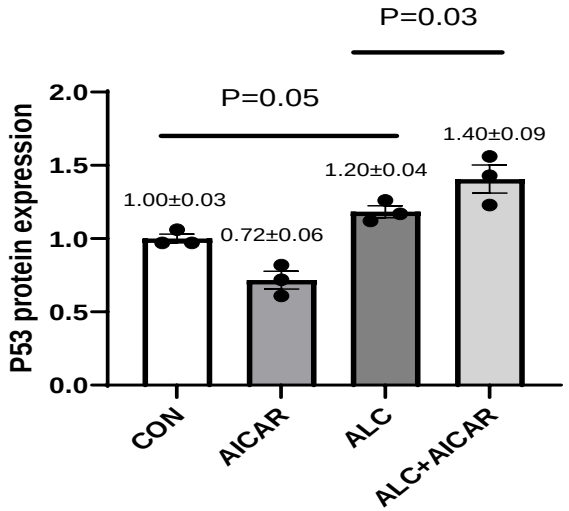
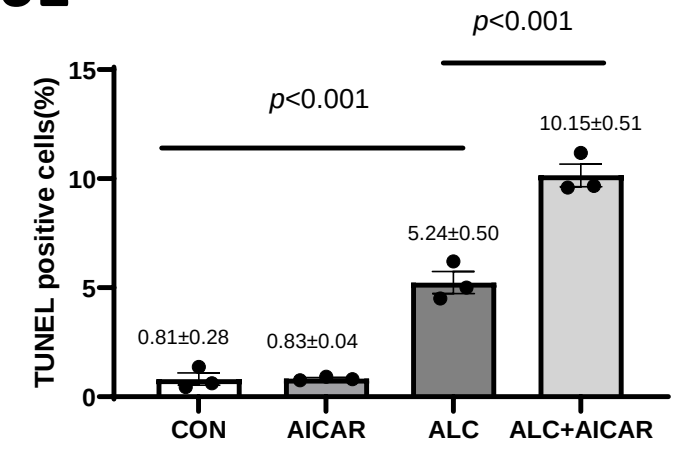


Figure 6C-E. The AMPK activator AICAR exacerbates ALC-mediated p53 stabilization and apoptosis, but is not sufficient to suppress rRNA synthesis or initiate p53-mediated apoptosis.







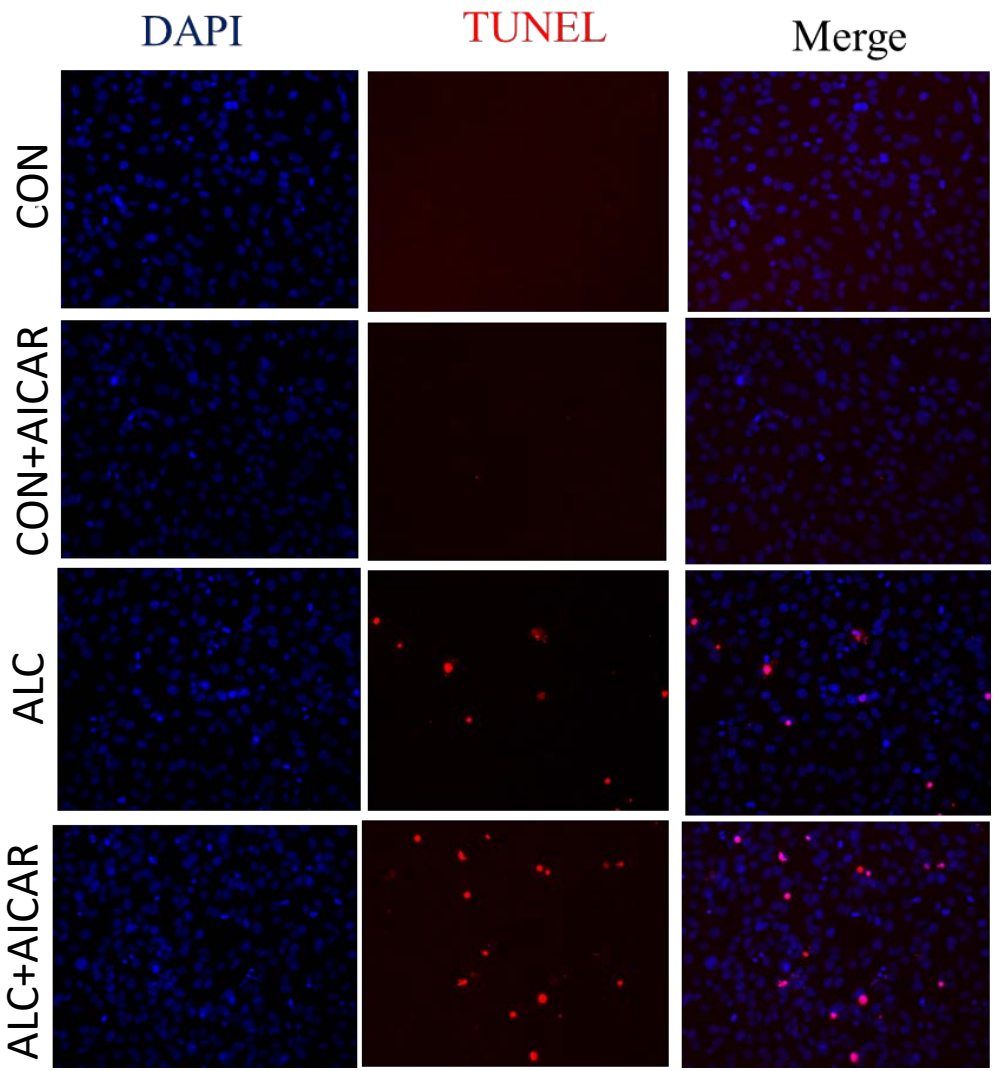
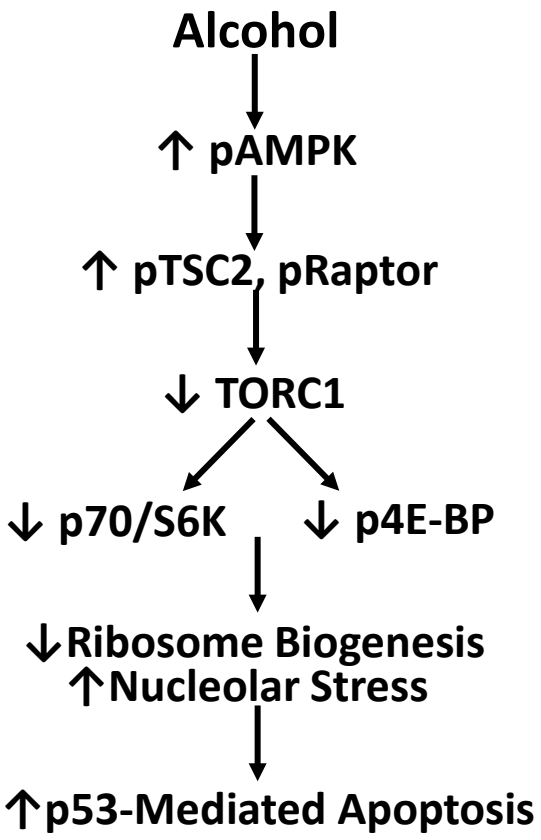



Figure 7. Mechanism of alcohol's action on neural crest.