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Report

A Mitochondrial Ribosomal and RNA Decay Pathway Blocks Cell Proliferation

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Summary

Proliferating cells require coordinated gene expression between the nucleus and mitochondria in order to divide, ensuring sufficient organelle number in daughter cells [1]. However, the machinery and mechanisms whereby proliferating cells monitor mitochondria and coordinate organelle biosynthesis remain poorly understood. Antibiotics inhibiting mitochondrial translation have emerged as therapeutics for human cancers because they block cell proliferation [2, 3]. These proliferative defects were attributable to modest decreases in mitochondrial respiration [3, 4], even though tumors are mainly glycolytic [5] and mitochondrial respiratory chain function appears to play a minor role in cell proliferation in vivo [6]. Here we challenge this interpretation by demonstrating that one class of antiproliferative antibiotic induces stalled mitochondrial ribosomes, which triggers a mitochondrial ribosome and RNA decay pathway. Rescue of the stalled mitochondrial ribosomes initiates a retrograde signaling response to block cell proliferation and occurs prior to any loss of mitochondrial respiration. The loss of respiratory chain function is simply a downstream effect of impaired mitochondrial translation and not the antiproliferative signal. This mitochondrial ribosome quality-control pathway is actively monitored in cells and constitutes an important organelle checkpoint for cell division.

Results and Discussion

In mammals, translation of the 13 mitochondrially encoded polypeptides occurs on a dedicated set of mitochondrial ribosomes (mito-ribosomes) with mechanistic features more similar to those of prokaryotes than to cytoplasmic ribosomes [7]. Initiation of mitochondrial protein synthesis requires a formylated methionine-tRNA [8]. Factors for the removal of the formyl group and starter methionine, as part of the N-terminal methionine excision (NME) pathway, are functional in mitochondria [9, 10]. Peptide deformylase (Pdf) initiates the pathway catalyzing removal of the formyl group from the starter methionine as the polypeptide chain emerges from the ribosomal exit tunnel. Pdf activity can be inhibited pharmacologically with peptide mimetics of the actinonin class, which are being developed as antiproliferative agents for human tumors [2]. Chloramphenicol is another well-known inhibitor of mitochondrial translation elongation, occupying the ribosomal A-site [11, 12], but with quite a different effect on cell proliferation [13].

Incubation of immortalized mouse embryonic fibroblasts (MEFs) with these antibiotics produced an expected time-dependent loss of mitochondrial respiratory chain components of differing severity (Figure 1A). Chloramphenicol decreased the mitochondrially encoded core subunit of cytochrome oxidase mt-Co1 to almost undetectable levels by 48 hr, whereas the effect with actinonin was modest in comparison (Figure 1A). The assembly of complex I was also impaired in proliferating MEFs (Figure 1A). The penetrance of these two antibiotics on mitochondrial protein synthesis differs and could be due to their mode of action. However, a striking feature of the actinonin treatment was loss of mito-ribosomal proteins, from both the small and large ribosomal subunits (Figures 1A and 1B). Depletion of mito-ribosomal proteins was time dependent (Figure 1B), reversible (Figure 1C), and not due to prior instability of the large and small mito-ribosomal subunits (Figure 1D). In contrast, chloramphenicol had no effect on the abundance of mito-ribosomal protein (Figure 1A). Moreover, this mito-ribosomal depletion after actinonin treatment is a conserved mitochondrial response to this antibiotic stress as shown by the fact that we also observed the same result in proliferating human foreskin cells and a cancer cell line (HeLa) (Figure S1 available online).

Mutations in human mito-ribosomal proteins and defects in mitochondrial rRNA synthesis or processing can impair the steady-state level of assembled mito-ribosomal subunits [14–17]. This effect is specific for either the large or small subunit, not both subunits simultaneously, and has no adverse effects on cell proliferation. Actinonin induced a time-dependent loss of both the mitochondrial 12S and 16S rRNA, matching the loss of mito-ribosomal subunits (Figure 1E). Surprisingly, there was also depletion of mitochondrial mRNA transcripts from both the light strand and heavy strand (Figure 1E). In proliferating cells, mitochondrial RNA species have relatively short half-lives (25 to 210 min depending upon the RNA) [18] but can be stabilized with the loss of mitochondrial transcription to sustain translation of mitochondrial proteins for up to 48 hr [19]. Actinonin had no effect on mitochondrial tRNA levels (Figure 1F), and mtDNA (Figure 1G) was also unaffected, suggesting that maintenance and transcription of the mitochondrial genome was not altered. Two other mitochondrial translation inhibitors, chloramphenicol and doxycycline [20], had no affect on mitochondrial RNA levels (Figure 1H), indicating that mitochondrial translation inhibition per se did not initiate this specific RNA decay. These results indicate that actinonin incubation leads to the specific degradation of mito-ribosomes and the rRNA and mRNA pool in a time-dependent manner.

Actinonin and chloramphenicol had a striking difference on cell proliferation that did not correlate with the severity of the mitochondrial respiratory chain reduction. Actinonin produced a progressive growth arrest, whereas chloramphenicol had no

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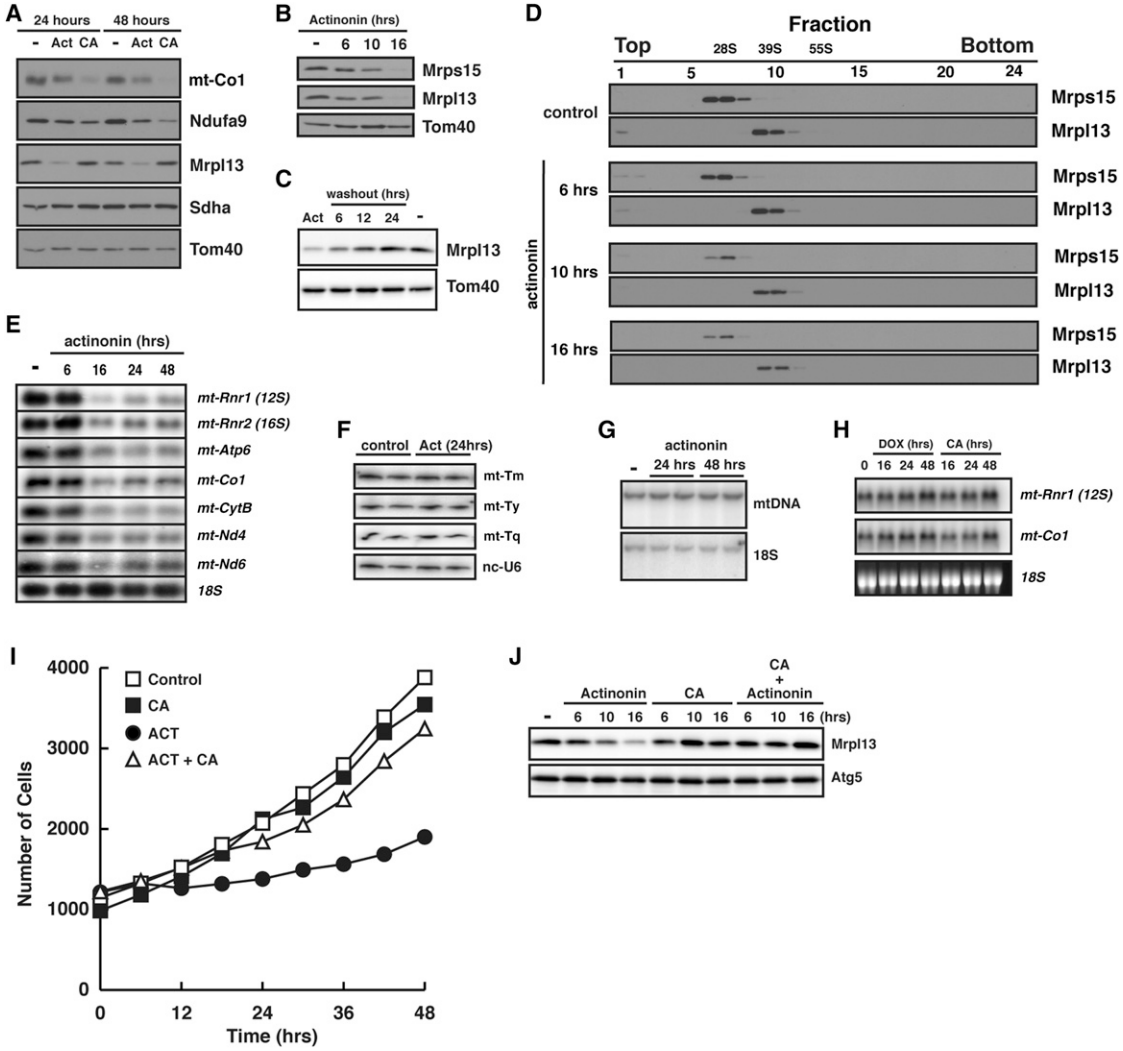

Figure 1. Actinonin Induces Acute Depletion of Mitochondrial Ribosomes, rRNA, and mRNA and Blocks Cell Proliferation

(A) Actinonin (Act) induces loss of mitochondrial ribosomal protein with a modest decrease of mitochondrial respiratory chain complexes in contrast to the mitochondrial translation inhibitor chloramphenicol (CA). Immunoblots of MEF total cell lysates treated with antibiotics.

(B) Time-dependent decrease of both large and small mitochondrial ribosomal proteins with actinonin treatment. Immunoblots of MEF total cell lysates treated with actinonin.

(C) Recovery of mito-ribosomal protein after actinonin removal. Immunoblots of MEF total cell lysates treated with actinonin for 16 hr, then incubated with fresh medium for the marked times. Left lane (Act) with no washout; right lane (-) untreated control.

(D) Immunoblotting of fractions from linear 10%–30% sucrose density gradients.

(E) Northern blot of total RNA from MEFs treated with actinonin.

(F) Northern blot of mitochondrial tRNAs.

(G) Southern blot of MEF mtDNA.

(H) Northern blot of total RNA from MEFs treated with chloramphenicol or doxycycline for the indicated times.

(I) MEF growth curves with antibiotics. Representative growth curves from three independent experiments.

(J) Immunoblot of MEF total cell lysates after antibiotic treatment.

effect on cell proliferation (Figure 1I). Importantly, cotreating cells with both actinonin and chloramphenicol suppressed the growth arrest (Figure 1I) and depletion of mito-ribosomal protein (Figure 1J), suggesting that these phenotypes are linked, independent of mitochondrial respiratory chain dysfunction, and due to mito-ribosome stress and not off-target effects of actinonin.

Our data indicate that there is a sudden loss of the mitochondrial translation apparatus in tandem with the antiproliferative effect of actinonin. We next tested whether de novo mitochondrial protein synthesis was impaired preceding this ribosomal and RNA decay by specifically radiolabeling the 13 mitochondrially encoded polypeptides [21]. After only 2 hr of treatment, there was a 40% decrease in the rate of mitochondrial translation, which was almost completely abolished after 6 hr (Figure 2A). This progressive inhibition of all mitochondrial protein synthesis could arise from impaired deformylation of nascent polypeptide chains or stalled mito-ribosomes. However, mass-spec analysis of the N termini from mitochondrial polypeptides in the heart indicates that only mt-Co3 undergoes deformylation [22] and thus would not account for the observed translation defect (Figure 2A).

How could actinonin induce stalled mito-ribosomes? In bacteria, Pdf is not a structural component of the ribosome,

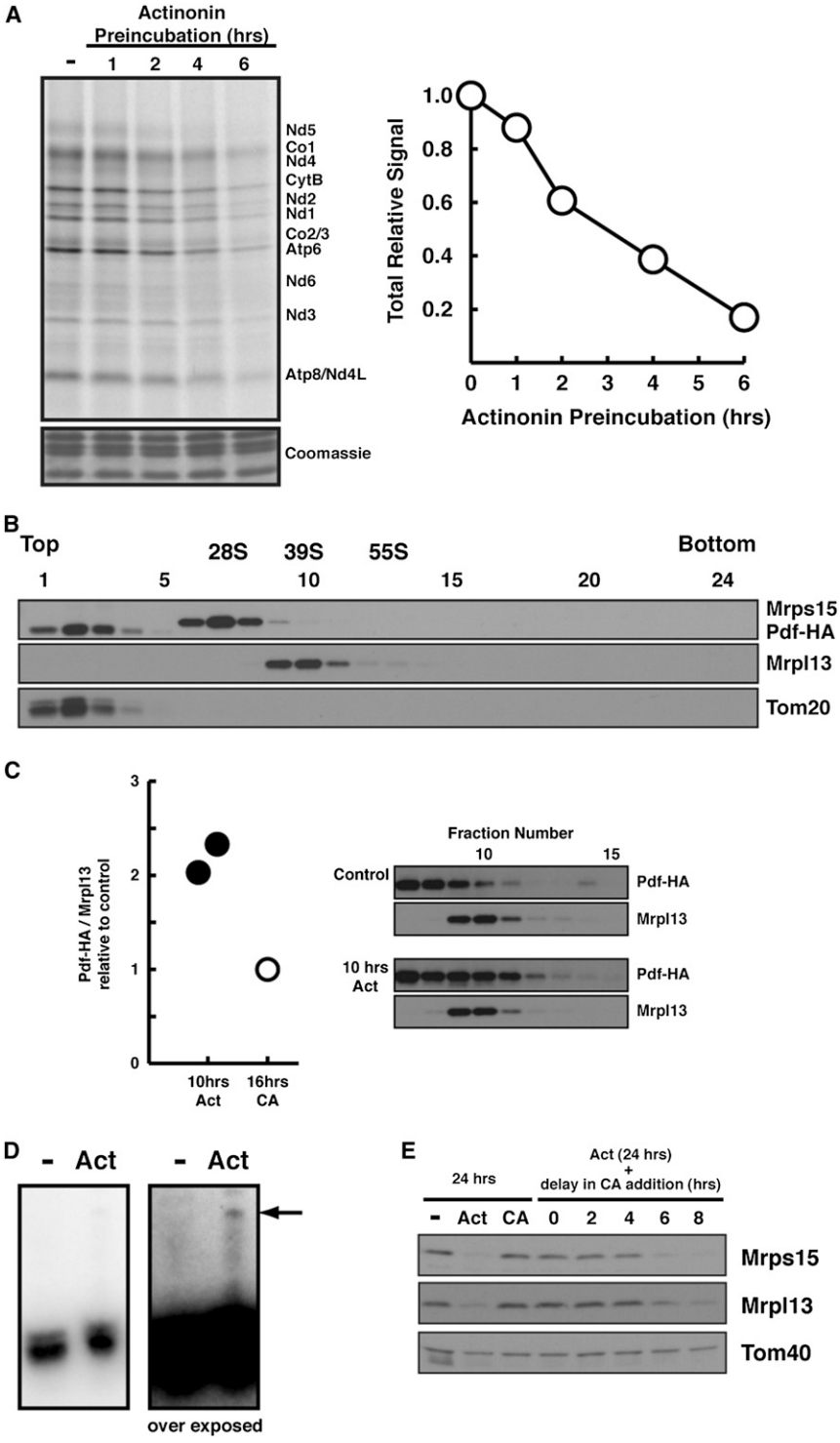

Figure 2. Actinonin Induces Stalling of Mitochondrial Ribosomes

(A) Synthesis of mitochondrial polypeptides in MEFs preincubated with actinonin for the indicated time and then pulsed with ^{35}S-Met/Cys. Right, quantification of the total signal from all subunits except Nd4 and Nd6.

(B) Immunoblotting of fractions from a linear 10%–30% sucrose density gradient of MEFs stably expressing HA-tagged peptide deformylase.

(C) Quantification of HA-tagged peptide deformylase accumulation in fractions from sucrose density gradients where the large ribosomal subunit sediments. The data represent two independent experiments. Extended incubation with chloramphenicol (CA) does not affect the abundance of Pdf on large subunit. On the right, representative film exposure showing Pdf-HA accumulation with actinonin.

(D) Northern blot of total RNA after acidic urea-PAGE from MEFs probed with oligos for the mitochondrial tRNA$^{Leu(CUN)}$ and tRNA$^{Ser(UCN)}$ showing accumulation of peptidyl-tRNA (arrow) with 3 hr of actinonin treatment.

(E) Immunoblot of MEF total cell lysates after antibiotic treatment.

but instead rapidly binds and dissociates from the ribosome surface to sample nascent chains [23]. Mitochondrial Pdf binds actinonin through an induced fit, similarly to its endogenous substrates except the antibiotic traps the enzyme in a final key-lock state [24]. If actinonin trapped Pdf on the large mito-ribosomal subunit near the exit tunnel, this situation could generate a steric hindrance to the emergence of nascent polypeptide chains and account for the generalized translation defect observed (Figure 2A). Notably, stable expression of HA-tagged Pdf in MEFs showed that most of the enzyme did not associate with either mito-ribosomal subunits or the monosome (Figures 2B and S2; Table S1). However, actinonin treatment leads to a specific increase in the amount of Pdf cosedimenting with the large ribosomal subunit, whereas chloramphenicol had no effect (Figure 2C). Chloramphenicol also stalls mito-ribosomes, by binding to the ribosomal A-site and arresting translation at random positions along the mRNA, but with clearly different consequences to mitochondrial and cellular homeostasis.

Under such a model, stalled nascent chains emerging from the exit tunnel would generate a temporary accumulation of polypeptidyl-tRNAs trapped in the P-site [25] before ribosome quality-control pathways are triggered [26]. The estimated length of the mito-ribosomal exit tunnel is 88 Å [27] and would correspond to a minimum 26 amino acids (assuming 3.35 Å per amino acid) before tertiary folding of the nascent chain [28]. Within the first 20 to 40 amino acid positions of all 13 mitochondrial polypeptides, leucine and serine are significantly enriched (Figure S3). To test for the accumulation of polypeptidyl-tRNA, we probed northern blots of total RNA (which contain polypeptidyl-tRNAs with less than 80 amino acids [25]) with oligos against tRNA$^{Leu(CUN)}$ and tRNA$^{Ser(UCN)}$ (Figures 2D and S3). Actinonin treatment led to the accumulation of a peptidyl-tRNA (Figure 2D). Although the abundance of this peptidyl-tRNA relative to the charged tRNA is low, it is nonetheless consistent with the observation that proliferating cells have

very little monosome (Figure S2). However, other factors could also play a role. These data suggest that actinonin impairment of Pdf leads to mito-ribosome stalling and precedes the abrupt degradation of mito-ribosomes and RNA. The specificity of this actinonin-induced translation stress may be a trigger for the decay pathway.

Rescue mechanisms for stalled ribosomes exist as part of conserved RNA surveillance pathways found in prokaryotes and eukaryotes, although so far not documented for organellar ribosomes [26, 29]. The depletion of mitochondrial rRNA, mRNA, and mito-ribosomes with actinonin are features observed in no-go decay (NGD) and nonfunctional ribosomal decay (NRD) described for cytoplasmic ribosomes [26, 29]. These quality-control pathways can initiate by stalled ribosomes from either defects in RNA folding or nascent polypeptide chains in the ribosome [26]. However, ribosomes are energetically expensive to synthesize. Initially a rescue pathway dissociates the stalled monosome to reuse the assembled small and large subunits, but in some cases ribosomes cannot be salvaged and are degraded by the proteasome [26, 29]. To initiate this rescue requires access to the ribosomal A-site for translation release factors [26, 29].

Mitochondria have three putative class I translation release factors lacking codon specificity [30]. The fact that chloramphenicol blocks access to the ribosomal A-site and can suppress the actinonin-mediated growth arrest and mito-ribosomal decay indicates that the rescue of these stalled mito-ribosomes is prevented. However, there was a threshold for the chloramphenicol-mediated suppression effect, suggesting that blocking translation per se was not sufficient to prevent the ribosomal and RNA decay (Figure 2E). Actinonin incubation for up to 4 hr can lead to a 60% decrease in mitochondrial protein synthesis (Figure 2A) and yet subsequent administration of chloramphenicol can prevent the ribosomal decay (Figure 2E). Beyond 4 hr of actinonin incubation, the chloramphenicol suppression was lost (Figure 2E) even though between 4 and 6 hr of actinonin treatment there was no detectable loss of RNA or mito-ribosomal proteins (Figures 1B and 1E). These data are consistent with the idea that damage or stress accumulates from rescuing the stalling and when this level reaches a certain threshold (after 4 hr of actinonin), a decay pathway initiates. Such a scenario has been postulated for stalling of cytoplasmic ribosomes in eukaryotes [26].

Mito-ribosomes are tightly associated with the inner mitochondrial membrane, an interaction mediated in part by prohibitins [31]. Loss of prohibitins generates fragmented mitochondria and defects in cell proliferation independent of cellular ATP levels and respiration [32]. We found that actinonin induced a time-dependent fragmentation of the complete mitochondrial network within 6 hr, whereas chloramphenicol had no effect (Figure 3A). Opa1 is an inner membrane GTPase important for membrane fusion and cristae organization and is proteolytically processed into five mature isoforms. Cell stress can induce cleavage of the membrane-anchored long isoforms (L1 and L2) and membrane fragmentation [33, 34]. Actinonin also induced loss of the long Opa1 isoforms (Figure 3B), consistent with the membrane fragmentation. However, blocking the rescue of those stalled mito-ribosomes by coincubating with chloramphenicol suppressed this Opa1 cleavage, indicating that membrane fragmentation arises as a consequence of the rescue pathway, not simply by loss of mitochondrial protein synthesis.

To confirm that actinonin required mito-ribosomes to induce the Opa1 cleavage, we retrovirally transduced MEFs with the UL12.5 viral gene [35], a mitochondrially targeted nuclease that eliminates mtDNA and as a result mito-ribosomes (Figure 3C). Loss of mito-ribosomes completely abrogated the actinonin effect on Opa1 cleavage (Figure 3D). Moreover, depletion of mito-ribosomes with UL12.5 also generated a severe growth defect insensitive to actinonin (Figure 3E), which clearly indicates the requirement of mito-ribosomes for these responses.

To block cell proliferation, a mito-ribosomal rescue pathway must trigger a retrograde signal from the organelle to the nucleus to coordinate the response. Mitochondrial retrograde responses have been identified in eukaryotes for misfolded proteins and mitochondrial dysfunction [36–38]. To obtain a more complete picture on the retrograde signaling and the downstream pathways responsive to this mito-ribosomal rescue, we analyzed the cellular RNA expression profile by microarray in MEFs treated with actinonin. After only 6 hr there were profound changes in nuclear gene expression (Figure 4A; Table S2). This initial response involves genes enriched in pathways for cell cycle regulation, p53 signaling, MAPK, cytosolic translation, and mitochondrial respiratory chain (Figure 4B). These pathways are critically regulated for cell proliferation [5]. These acute changes in gene expression were independent of the cytosolic energy sensor Ampk-alpha after 6 hr of actinonin (Figure 4C), suggesting initially that there was no cellular energy crisis (Figure S4). Overall, the kinetic of these early gene expression changes is consistent with the rescue of mito-ribosomes and precedes the degradation of mito-ribosomes and RNA, suggesting that the rescue pathway triggers a retrograde signal to modulate cell proliferation.

Our findings document an organellar ribosomal decay pathway actively monitored in dividing cells that can arrest cell proliferation when triggered by actinonin-induced stalling of mito-ribosomes. There are two components to this mechanism, one activated by the stalling and rescue of mito-ribosomes and the other initiating the ribosomal and RNA decay. Initially, stalled mito-ribosomes would be rescued potentially through mitochondrial release factors binding to the A-site. The kinetic of this rescue event (less than 6 hr) suggests that it initiates the retrograde signaling to halt cell proliferation, possibly mediated through remodeling of mitochondrial membranes (fragmentation and Opa1 processing). The observation that chloramphenicol specifically can suppress all of these effects on the same time scale supports the interpretation that actinonin-induced stalling triggers rescue of mito-ribosomes dependent upon the A-site. However, sustained stalling/rescue of mito-ribosomes is likely to generate a signal that activates this mitochondrial ribosomal and RNA decay pathway. Once this threshold has been reached, activation of the decay pathway cannot be stopped and ensures elimination of the majority of mito-ribosomes and mitochondrial rRNA and mRNA. Such a mechanism has been posited for cytoplasmic ribosomes [26]. The nature of this damage and retrograde signals remain to be determined.

In proliferating cells, this monitoring is probably proportional to mitochondrial abundance of the cell and probably accounts for the sensitivity of leukemia to actinonin [2]. This pathway indicates the existence of cell-autonomous regulation to cell proliferation by monitoring mitochondria through a process independent of energy metabolism and extracellular growth factors. The physiological relevance of this mito-ribosomal decay pathway would be important with mtDNA mutations, particularly with genome deletions or high mutation loads, which would predictably generate stalled mito-ribosomes

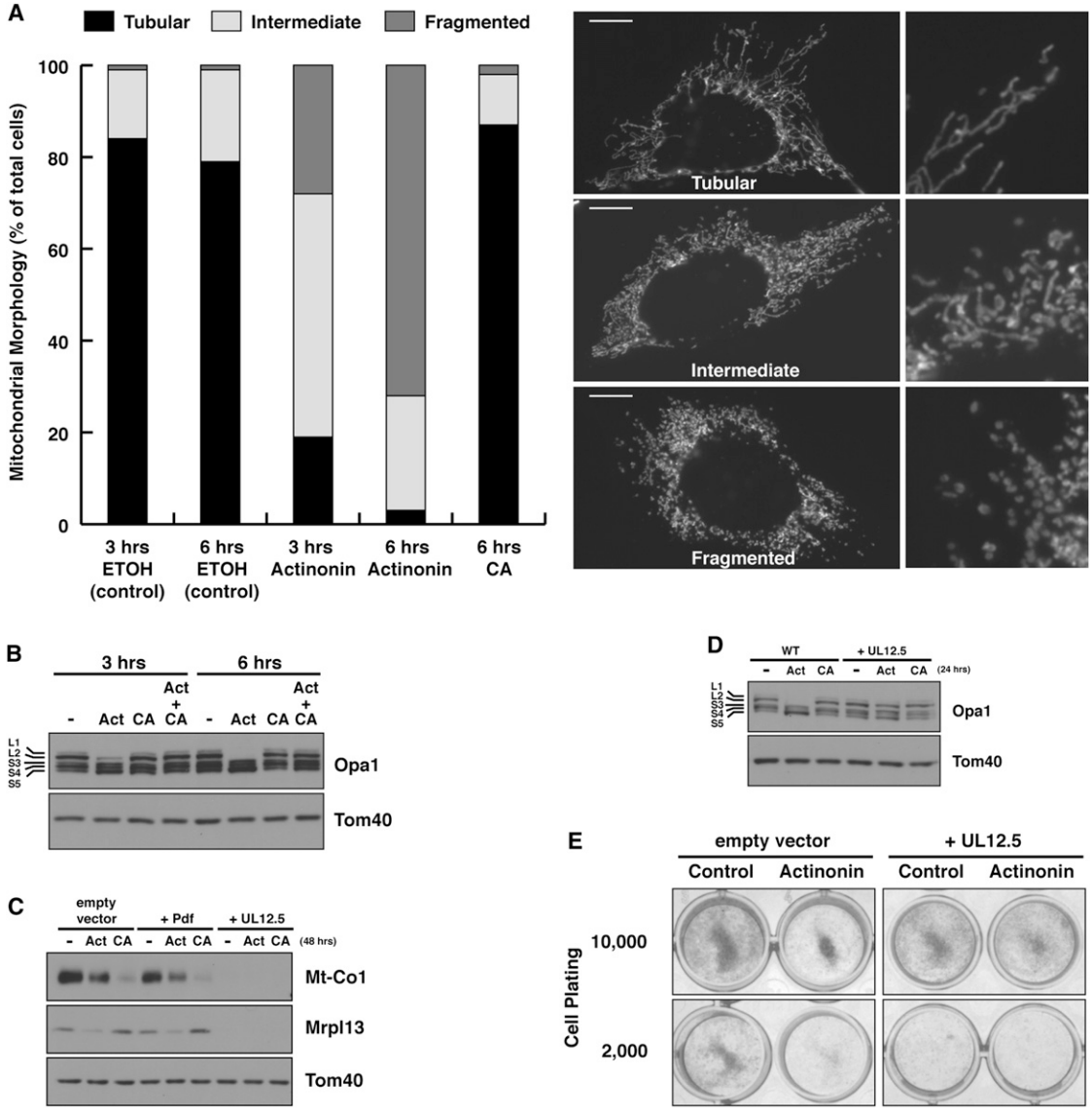

Figure 3. Rescue of Stalled Mitochondrial Ribosomes Fragments Mitochondrial Membranes

(A) MEFs were transfected with GFP-Omp25 and then treated with actinonin (Act), chloramphenicol (CA), or carrier (ETOH). Mitochondrial morphology was classified into three categories (right, scale bars represent 10 μm). Results represent three independent experiments (n = 100 cells per treatment and experiment).

(B–D) Immunoblots of MEFs treated with antibiotics.

(B) Time-dependent proteolytic processing of Opa1 only with actinonin treatment, which can be suppressed by chloramphenicol.

(C) Expression of the viral UL12.5 nuclease eliminates mtDNA and the abundance of mitochondrial ribosomes.

(D) Expression of UL12.5 in MEFs prevents the actinonin-mediated proteolytic processing of Opa1.

(E) Crystal violet staining of MEFs retrovirally transduced with an empty vector or UL12.5 grown for 72 hr with or without actinonin.

and are known to affect proliferating cells of the hematopoietic lineage in humans and mice [39, 40]. It remains to be determined how such a rescue pathway would function in postmitotic cells, since these cell types have a higher number of mitochondria and are more dependent upon aerobic energy metabolism.

Experimental Procedures

Cell Culture, Retrovirus, Immunoblotting, and Mitochondrial Protein Synthesis

Mouse embryonic fibroblasts (MEFs) immortalized by retroviral transduction of E7/hTERT [41] were cultured under standard conditions with DMEM (Lonza) supplemented with 10% fetal bovine serum and 50 mg/ml uridine. Cells were treated with actinonin (150 μM), chloramphenicol (200 μg/ml), or carrier (100% ethanol). For crystal violet staining, cells were washed with PBS and fixed for 15 min in 100% methanol, followed by 0.1% crystal violet for 15 min. Growth curves were generated with the Cell-IQ monitoring culture platform and Cell-IQ Analyzer Pro-Write software (Chip-Man Technologies). Mouse peptide deformylase was cloned into the retroviral expression vector pBABE-puro, and UL12.5 (kind gift of James Smiley) was cloned into pMXs-IRES-Blasticidin. Retrovirus was generated by transient transfection into the Phoenix amphotropic packaging line. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by semi-dry transfer. Primary antibodies used: Mrpl13 and Mrps15 (kind gift of Nils Göran Larsson), Atg5 (Sigma), HA (Sigma), mt-Co1 (MitoScience), Ndufa9 (MitoScience), Sdha (MitoScience), Tom20 and Tom40 (Santa Cruz), Opa1 (BD), and Ampk-alpha and phospho(Thr172)-Ampk-alpha (Cell Signaling). ECL was used with film or a Chemi-doc imaging station (BioRAD)

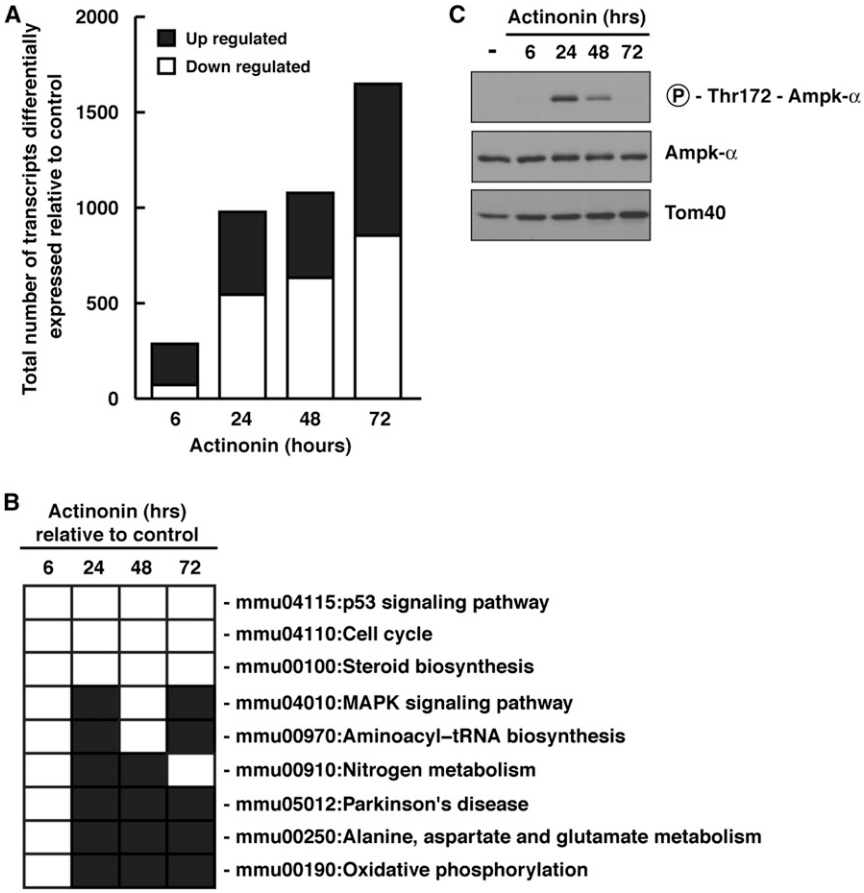

Figure 4. Rescue of Stalled Mitochondrial Ribosomes Remodels Nuclear Gene Expression to Halt Cell Proliferation

(A) Differentially expressed genes by microarray analysis with actinonin relative to the control (n = 4, for each time point and the control). Inclusion criteria was at least a 1.5-fold linear change and $p < 0.01$ after Benjamini and Hochberg correction.

(B) KEGG pathway analysis for differentially expressed genes with actinonin treatment relative to the control. White boxes indicate pathways significantly enriched for differentially expressed genes (minimum two genes in a pathway, Fisher test $p < 0.10$) with actinonin treatment compared to the control.

(C) Immunoblotting of MEFs treated with actinonin.

for signal detection. Representative immunoblotting data were cropped in Photoshop with only linear corrections applied. Pulse labeling of mitochondrial protein synthesis was performed as described [21] with gels rehydrated for 4 hr in PBS and Coomassie stained to confirm equal loading.

Southern and Northern Blotting and Microarray

Total DNA was isolated from MEFs and Southern blot as described [42]. Total cellular RNA was isolated with Trizol (Invitrogen) and separated on 1.2% agarose-formaldehyde gels, transferred to Hybond-N^+ membrane (GE Healthcare) by neutral transfer, and then hybridized to 5′ radiolabeled oligonucleotides probes to detect mitochondrial transcripts. Mitochondrial tRNAs were detected from total RNA separated on 7M urea-PAGE and hybridized with radiolabeled probes. Gene expression analysis was performed with the Agilent SurePrint G3 Mouse GE 8 × 60K Microarrays. A total of four independent samples were used for each actinonin-treated time point and control. Each individual sample (n = 1) consisted of RNA extracted from cells of four pooled 10 cm plates.

Isokinetic Sucrose Gradient Assays

MEFs or isolated mitochondria were lysed (50 mM Tris [pH 7.2], 10 mM $Mg(Ac)_2$, 40 mM NH_4Cl, 100 mM KCl, 1% DDM, and 1 mM PMSF) for 20 min on ice followed by centrifugation for 20 min at 20,000 × *g* at 4°C. The supernatant was loaded on top of a 16 ml linear 10%–30% sucrose gradient (50 mM Tris [pH 7.2], 10 mM $Mg(Ac)_2$, 40 mM NH_4Cl, 100 mM KCl, and 1 mM PMSF) and centrifuged for 15 hr at 4°C and 74,400 × *g* (SW 32.1 Ti). 24 equal volume fractions were collected from the top and TCA precipitated. Samples were separated by SDS-PAGE for immunoblotting or liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Fluorescence Microscopy

MEFs were transiently transfected with GFP-omp25 [43], then treated on the following day with antibiotics or ethanol. Cells were fixed in 4% paraformaldehyde and mounted with DABCO/MOWIOL on glass slides for imaging on a Zeiss Axioplan2 with a 100× (1.3 NA) objective.

Accession Numbers

The GEO accession number for the microarray data is GSE44109.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.019.

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