Determining the off-target activity of antibiotics and novel translation initiation sites in mitochondria

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Abstract

Mitochondria translate the 13 mitochondrially encoded proteins using a dedicated translation system that closely resembles the one found in prokaryotes. Consequently, many bacterial protein synthesis inhibitors, used as antibiotics, exhibit mitochondrial toxicity as off-target effects. However, it remains unclear whether these antibiotics act through the same mechanisms in mitochondria as in bacteria. To address this, we characterized the effects of a panel of bacterial translation and elongation inhibitors on mitochondrial translation through mitoribosome profiling. We found that the mechanism of action for every antibiotic, except telithromycin, is the same in both bacteria and mitochondria. Additionally, mitoribosome profiling data showed that *MT-ND1* and *MT-ND5* have incorrectly annotated translation initiation sites and suggested several novel mitochondrial translation events. Careful study of the mechanisms of mitochondrial translation inhibition can guide development of antibiotics with increased target specificity and reduced mitochondrial toxicity.

Introduction

Mitochondria contain their own genome, which in mammals canonically encodes thirteen proteins. These proteins are essential building blocks of complexes I, III, and IV of the electron transport chain as well as ATP synthetase. They are synthesized by a dedicated translation system localized to the mitochondrial matrix that is conserved from the ancestral bacteria that evolved into the present-day organelles through an endosymbiotic event[1]. As a result, mitochondrial translation is wholly distinct from cytoplasmic translation and utilizes its own mitochondrial ribosomes (mitoribosomes), tRNAs, and translation factors. The RNA components of the machinery are mitochondrially encoded, while its protein components are encoded in the nucleus, translated by the cytoplasmic ribosome, and subsequently imported into the mitochondria[2].

Chronic impairment of mitochondrial translation, most commonly through mutations in translation factors, results in disrupted mitochondrial function and causes diseases such as MERRF (Myoclonic Epilepsy with Ragged-Red Fibers)[3], MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes)[4], and maternally inherited deafness[5,6]. Furthermore, acute inhibition of mitochondrial translation caused by the off-target action of antibiotics targeting the bacterial ribosome can also cause mitochondrial toxicity[7-10]. This toxicity can manifest as lactic acidosis, ototoxicity[11], nephrotoxicity[12], and peripheral neuropathy[13], depending on the specific antibiotic and the genetic background of the recipient[14]. While the United States has worked to regulate antibiotics with severe side effects, they remain a global issue. For example, chloramphenicol, whose use is discontinued in the United States, causes fatal aplastic anemia at a rate of 1 in 60,000[7] but remains widely prescribed in developing countries due to its low cost to produce[15,16]. Surveys suggest that approximately 14.3 doses of antibiotics are administered per 1,000 people every day worldwide[17]. The toxic effects antibiotics can be exacerbated when taken without medical supervision. In addition, safety concerns surrounding drugs like chloramphenicol and telithromycin, which is linked to liver failure[18], limits our pool of available antibiotics while we are faced with the rise of multidrug-resistant bacteria. Studying the off-target effect of these drugs can guide the development of safer therapies.

Multiple classes of antibiotics inhibit bacterial translation by binding functionally important site on the ribosome and disrupting a specific process in translation, resulting in target ribosome arresting translation on mRNAs at sites directly related to the mechanism of inhibition[19,20]. In some instances, this relationship is straightforward. For example, the pleuromutilin antibiotic retapamulin binds to the peptidyl-transferase center (PTC) of the bacterial ribosome and impedes translation initiation by interfering with positioning of initiator tRNA[21], resulting in bacterial

ribosomes accumulating at start codons[22]. Inhibition by other antibiotics is more complicated, only disrupting translation when specific sequences are being translated, called context-dependent translation arrest. This is exemplified by chloramphenicol, which simultaneously interacts with the nascent peptide and the peptidyl-transferase center at its' binding site and disrupts accommodation of aminoacyl-tRNA, thereby acting as an elongation inhibitor[23]. Due to chloramphenicol's interaction with the nascent peptide, it selectively causes ribosomes to arrest translation when an alanine, serine, or threonine in the –1 position of the nascent peptide[24]. Additionally, chloramphenicol does not interfere with accommodation of glycine due to its' minimal size. The mechanisms of these and other antibiotics have been determined by ribosome profiling in bacterial cells. However, it remains unclear whether chloramphenicol or other antibiotics inhibit mitochondrial using identical mechanism.

To determine the sites of translation arrest we utilized mitoribosome profiling in HEK293 cells treated with antibiotics[18,25]. Ribosome profiling allows for the mapping of translating ribosomes with codon level accuracy by quantifying mRNA sequences occupied by translating ribosomes. To do this, RNase treatment degrades mRNA unprotected by the ribosome to generate ribosome protected fragments (RPFs). Because cytoplasmic ribosomes significantly outnumber mitoribosomes, mitoribosomes containing the mitochondrial RPFs (MRPFs) are isolated through sucrose gradient fractionation, where the eukaryotic ribosome and mitoribosome are sediment as 80S and 55S fractions respectively (Figure 1A). RPFs are isolated and quantified by next-generation sequencing. Increases in mitochondrial MRPFs reveal the specific codons and sequence contexts prevalent at sites where mitochondrial translation arrests due to antibiotic treatment.

Understanding the mechanisms of translation inhibitors in mitochondria can guide development of therapeutics that are more selective to the bacterial ribosome and lays the groundwork for determining how these therapeutics disrupt mitochondrial function. In this study we utilized mitoribosome profiling, a method for mapping the locations of translating mitoribosomes with codon level accuracy, in the presence of the bacterial protein synthesis inhibitors retapamulin, tiamulin, josamycin, erythromycin, chloramphenicol, and linezolid to determine their mechanism of action. We found that the mechanism of mitochondrial inhibition is the same as bacterial inhibition for all inhibitors except telithromycin. While telithromycin selectively arrests bacterial ribosomes when either arginine (R) or lysine (K are in the –1 position of the nascent chain and A-site, called an R/K-X-R/K motif[26], it instead arrests mitochondrial translation at an R/K/A-X-K motif, suggesting an underlying difference in the mechanism in the

two systems. Additionally, nucleotide-level precision of mitoribosome profiling also allowed us to precisely annotate the mitochondrial gene structure. We found that *MT-ND1* and *MT-ND5* genes use alternative translation initiation sites and identified a putative open reading frame on the *MT-RNR1* gene. This work demonstrates that mitoribosome profiling is an effective tool to study the fundamentals of mitochondrial translation and the mechanisms of translation impairment affecting human health.

Results

RNase I generates highly phased mitoribosome footprints

To optimize mitoribosome profiling, we tested two different RNases that were typically used for ribosome footprinting, RNase I and MNase, followed by isolation of the 55S mitoribosome by sucrose gradient fractionation. While both libraries are enriched for reads ~30 nucleotides (nt) in length, MNase footprints showed a bimodal distribution, with a clear secondary peak at ~22 nts (Figure 1C) indicating that MNase treatment may have captured a unique conformation of the mitoribosome. We mapped the sequence reads to the mitochondrial genome and its thirteen open reading frames (ORFs). For each mapped sequence read, we inferred the position of the ribosomal P-site using a 13nt offset from the 5'end, determined by the analysis of footprint distribution surrounding the initiation sites of *MT-ND4*, *MT-ND6*, and the newly reported *MT-ND5-dORF*. We observed coverage across mitochondrial ORFs, suggesting we have captured translating mitoribosomes (Figure 1B). Furthermore, analysis of RPF lengths revealed a distinct difference between RNase I and MNase digested footprint. While both libraries are enriched for reads ~30 nt in length, MNase footprints showed a bimodal distribution, with a clear secondary peak at ~22 nts (Figure 1C), which may indicate MNase may capture two distinct conformations of the ribosome. To determine the quality of the ribosome profiling data, we calculated read phasing, which occurs when an nuclease creates uniform MRPFs and the resulting ends of the MRPFs reflect the 3 nt periodicity of elongating ribosomes. While both datasets do show clear phasing, the read phasing is more biased toward position 1 in the RNase I-treated sample indicating slightly more consistent digestion (Figure 1D). Nevertheless, we observed similar levels of footprint abundance per gene, with the only notable exceptions being RNase I digested footprints were enriched on *MT-ND3* and depleted on *MT-ND4L* and *MT-ND6* (Figure 1E). While the cause of the difference is unclear, we did not expect it to interfere when comparing an antibiotic treated sample to a control. Read coverage also appeared similar with the two methods, with 85%-87% codons having between 2 and 50 reads mapping to them (Figure 1F). Overall, these results indicated that both MNase and RNase I digestion capture mitoribosome footprints with only minor differences. Considering the improved read phasing, we selected RNase I for our mitoribosome profiling experiments.

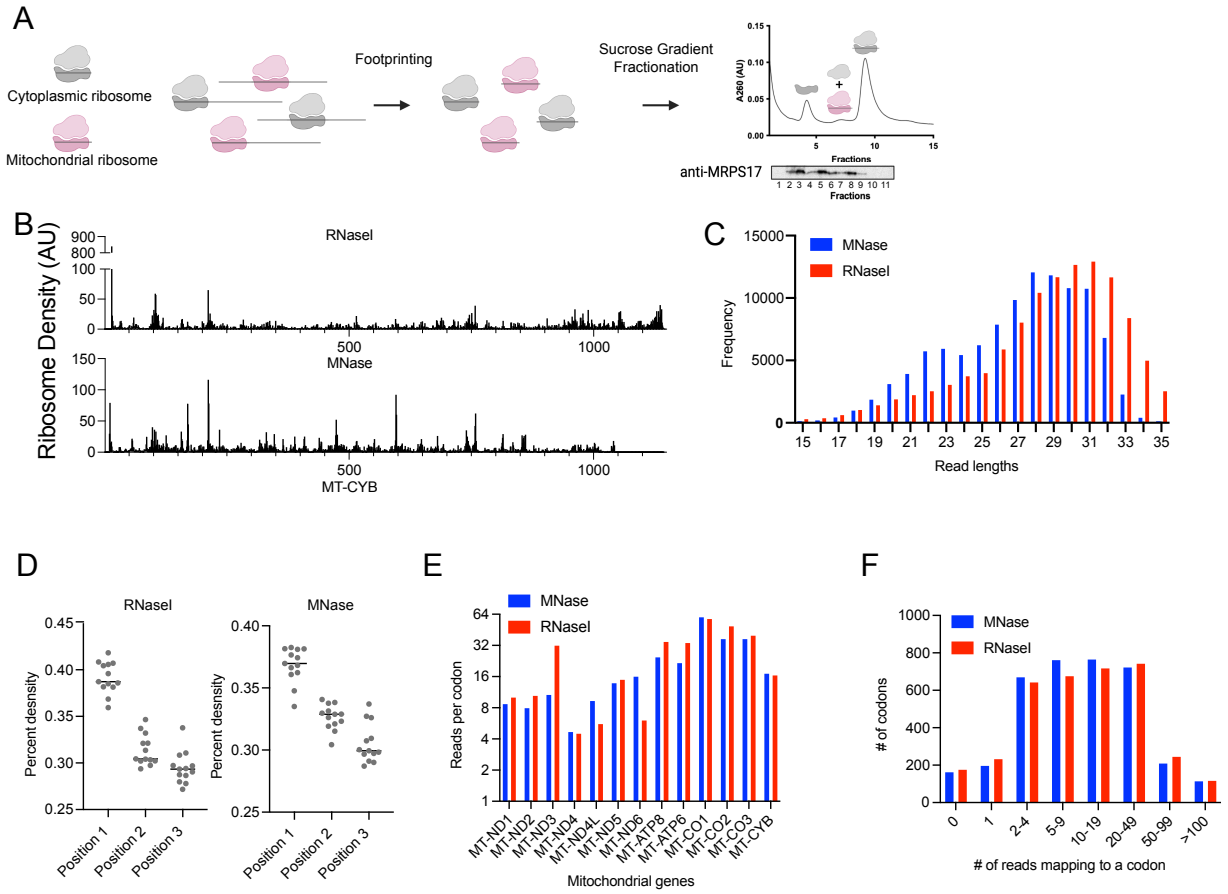

Figure 1. Mitoribosome profiling captures elongating ribosomes.

(A) Schematic representation for how translating mitoribosomes are footprinted and separated from cytoplasmic ribosomes by differential centrifugation. (B) Coverage of MRPFs on *MT-CYB* from mitoribosome profiling generated by either RNase I or MNase footprinting. (C) Distribution of read lengths of MRPFs mapping to the mitochondrial genome. (D) Read phasing analysis of MRPFs, where the 5' ends of reads are grouped by their subcodon position. Each dot represents an individual mitochondrial gene. (E) Average coverage of mitochondrial genes, calculated by dividing the total number of reads mapping to an ORF and dividing by the length of the ORF. (F) Distribution of mitochondrial codons grouped by the number of mitoribosome P-sites assigned to them.

Retapamulin, Tiamulin, and Josamycin inhibit translation initiation

First, we examined the impact of three antibiotics – retapamulin, josamycin, and tiamulin on inhibiting translation initiation on mitochondrial translation. Retapamulin and tiamulin are members of the pleuromutilin class of antibiotics. They work by binding to the P-site of the peptidyl transferase center (PTC) of the bacterial ribosome and disrupting initiator tRNA positioning (Figure 2A, left)[21,22]. Josamycin is a macrolide antibiotic that binds in the nascent peptide exit tunnel near the PTC and arrests bacterial ribosomes at initiation by interfering with A-site tRNA positioning (Figure 2A, right)[27]. We sought to understand whether these antibiotics also target translation initiation in mitochondria. To this end, we treated HEK293 cells with high concentrations of these antibiotics (100 µg/ml of tiamulin and josamycin, 10 µg/mL of retapamulin) for 30 minutes, performed mitoribosome profiling, and compared the treated experiments to a DMSO treated control.

MRPFs were mapped to the 13 mitochondrial genes. On *MT-CYB* and all other genes, we observed that after treatment with antibiotics reads accumulated at the 5' end of the transcript and MRPFs across of the rest of the coding region were lost or decreased (Figure 2B). We quantified the percentage of MRPFs mapping at the 5' end relative to the first 200 nucleotides. Mitoribosome profiles from cells treated with a DMSO control contained ~10% of footprints at translation initiation sites, which increased to 30%-40% when treated with an initiation inhibitor. Additionally, we quantified the cumulative footprint abundance moving from the start to the stop codon (Figure 2D). While ribosome footprints are generally evenly spread across the open reading frame in the DMSO control, MRPFs from cells treated with initiation inhibitors were biased towards the 5' end of the of the open reading frame. Together, these results indicate that retapamulin, tiamulin, and josamycin to act inhibit mitochondrial translation initiation, likely by the same mechanism as they do in prokaryotes.

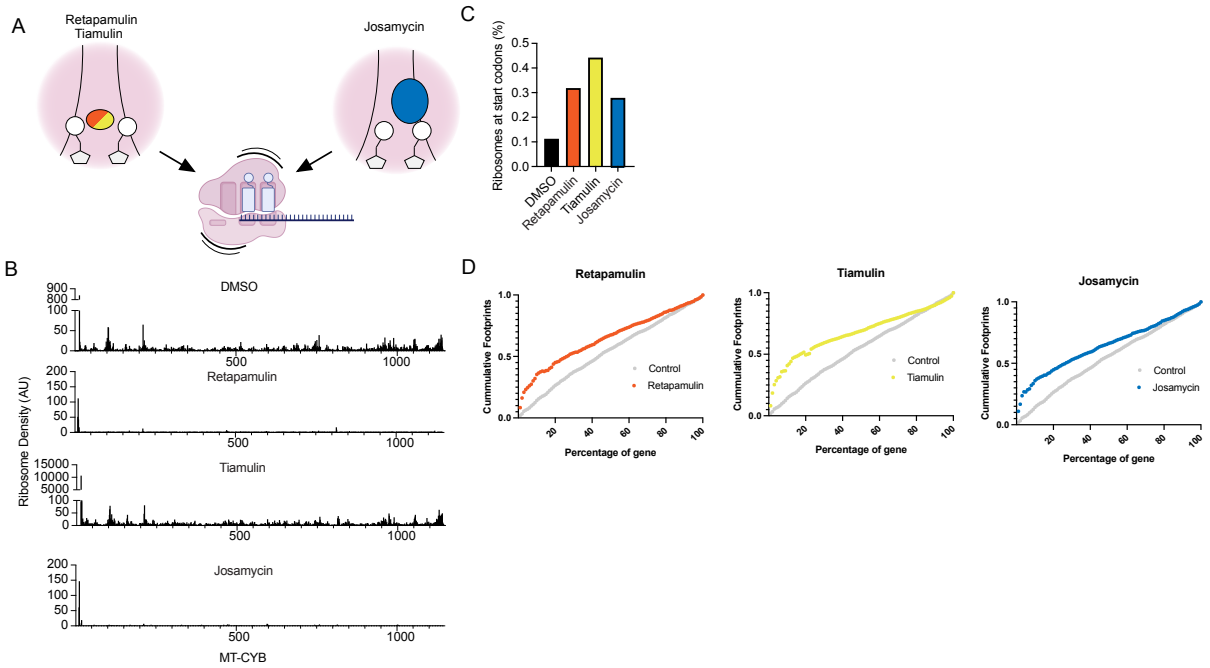

Figure 2. Inhibition of translation initiation in mitochondria.

(A) (Left) Schematic representation of retapamulin (orange) or tiamulin (yellow) binding to the PTC and disrupting the first round of elongation. (Right) Josamycin (blue) disrupts accommodation of the first aminoacyl-tRNA. All three inhibit initiation in bacteria. (B) MRPF coverage of *MT-CYB* treated with either DMSO, retapamulin, tiamulin, or josamycin. (C) Mean fraction of MRPFs in the first 200 nts each ORF that map to the 5' end of the transcript. (D) Cumulative distribution of ribosome density normalized across all mitochondrial ORFs.

Chloramphenicol, linezolid, and telithromycin arrest mitochondrial translation in a context-dependent manner.

Chloramphenicol, linezolid, and telithromycin selectively inhibit bacterial translation when ribosomes are translating specific sequences, in a process called context-dependent translation arrest[24,26]. Linezolid, like chloramphenicol, induces context-dependent arrest when alanine, serine, or threonine are at penultimate position of the nascent peptide (Figure 3A, left). Unlike chloramphenicol and linezolid, telithromycin binds in the nascent peptide exit tunnel near the PTC and adjacent to the nascent peptide[28]. During elongation, telithromycin causes ribosomes to arrest translation at a R/K-X-R/K motif, when the identity of both the penultimate position of the nascent peptide and the incoming aminoacyl-moiety are either arginine or lysine (Figure 3A right).

We carried out mitoribosome profiling on HEK293 cells treated with either antibiotic to understand whether these context-dependent translation inhibitors arrest mitoribosomes in the same manner as they arrest bacterial ribosomes. Unlike the cells treated with initiation inhibitors, cells treated with chloramphenicol or linezolid carried ribosome density throughout the entirety of the ORF, suggesting that, as expected, they were not acting as inhibitors of initiation (Figure 3B). Cells treated with telithromycin did show a slight increase in MRPFs abundance at the 5' end on *MT-CYB.* However, across all genes the levels of MRPFs at the 5' end were not near the levels of translation the initiation inhibitor retapamulin and were more similar to chloramphenicol, linezolid, and the DMSO control (Figure 3C). Cumulative distribution of MRPFs suggested that ribosome density was biased towards the 5' end of the gene for linezolid and telithromycin, but not significantly for chloramphenicol (Figure 3D). These results suggest that these three antibiotics are not acting as initiation inhibitors and that linezolid and telithromycin redistribute MRPFs to the 5' end of the ORF.

Next, we determined whether that redistribution of elongating ribosomes was dependent on the context of the sequence being translated. We calculated the average change of MRPFs depending on the identity of the amino acid at either the -1, P-site, or A-site positions. For both chloramphenicol and linezolid, we observed a specific increase in ribosome footprints when mitoribosomes encountered codons for alanine, serine, or threonine in the -1 position, with a 2-, 2.2-, and 1.3-fold change for chloramphenicol and a 2.3-, 1.4-, and 1.6-fold change for linezolid, respectively (Figure 3E). Additionally, we also observed context-dependent translation arrest induced by telithromycin. Unlike the R/K-X-R/K motif observed in bacteria, we found that telithromycin caused mitoribosomes to arrest at sites with a lysine positioned in the A-site, with an average 2.5-fold increase in MRPFs around those sites and that neither residue was enriched

in the -1 position. Due the limited size of mitochondrial genome there are only nine instances of the R/K-X-R/K motif, however, when we observed the positions with the greatest fold change, 3 of the top 4 contained the motif with lysine in the A-site (Figure 3F), suggesting that the motif was at least partially recapitulated. To specifically look for the motif we calculated the average fold change when we fixed arginine or lysine was in the A-site and varied the identity of the -1 amino acid (Figure 3G). We found that when lysine was in the A-site while either arginine, lysine, or alanine was -1 position, there was strong arrest (>5 fold change). However, when arginine was in the A-site we did not observe and strong stalling event with any –1 position residue. Therefore, in mitochondria, telithromycin induces stalling at R/K/A-X-K motifs. We concluded that the mechanism underlying context-dependent translation arrest for telithromycin is different in mitochondria compared to bacteria.

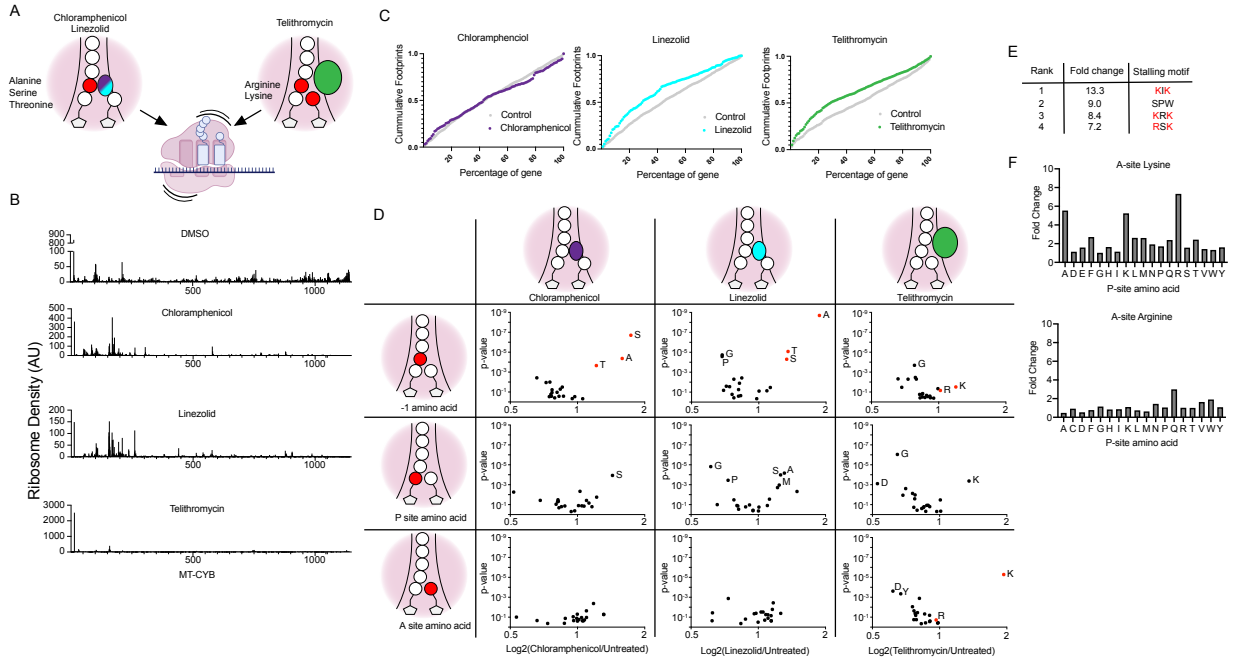

Figure 3. Context-dependent translation arrest by elongation inhibitors.

(A) Schematic representation of chloramphenicol (purple), linezolid (cyan), and telithromycin (green) inducing context-dependent translation arrest. (B) MRPF coverage of *MT-CYB* from cells treated with either DMSO, chloramphenicol, linezolid, or telithromycin. (C) Mean fraction of MRPFs in the first 200 nts each ORF that map to the 5' end of the transcript. (D) Cumulative distribution of ribosome density normalized across all mitochondrial ORFs. (E) Volcano plots of changes in MRPF fold change from cells treated with translation inhibitors. Each dot is the mean log2 fold change grouped by the identity of the –1 amino acid (top), P-site amino acid (middle) or A-site amino acid (right). (F) Top 4 strongest stalling sites induced by telithromycin treatment. Red amino acids indicate classical R/K-X-R/K stalling motif. G) Fold change in ribosome density, calculate as $2^{\text{mean(log2(Telithromycin/Control))}}$. Bars differ in the identity of the P-site amino acid and contain either a lysine in the A-site (top) or arginine (bottom).

Translation initiation on *MT-ND1 and MT-ND5* occurs at an alternative start codon

During the process of assigning the P-site positions of MRPFs, we observed that no ribosomes were assigned in the first 13 nucleotides of the 5' end of the transcript. This is not surprising, considering that mitochondrial transcripts often have no or very short 5' untranslated regions, typically less than 4 nucleotides in length and we applied a 13-nt offset from the 5' end of MRPFs. It reasons that as mitoribosomes begin translation on a leaderless mRNA, the 5' end of the resulting MRPF would have a full-length region 3' of the P-site and no sequence 5' of the P-site. As the ribosomes moves down the transcript, the 5' end of the MRPF would increase until it is accessible for digestion, at which point it would generate 31 nt-long MRPFs (Figure 4A). As accurate mapping of mitoribosomes at initiation could be a useful tool to understand mitochondrial translation, we attempted to map the location of initiating mitoribosomes at 5' end using footprint length, rather than 5' offset. Because mitoribosome profiling with MNase generated a population of ribosomes with short MRPFs, we hypothesized that these footprints could be indicative of capturing initiating ribosomes (Figure 1C). We plotted the distribution of MRPFs from 20 to 34 nt length that mapped to the 5' end of the 11 leaderless or short leader ORFs and compared them to reads mapping to the whole ORF. We observed that MRPFs 22-23 nts in length with 6-fold more abundant and MRPFs >28 nts in length were depleted compared to the whole ORF (Figure 4B). As mitochondrial ORFs vary in the length of their 5' UTR and we expect most ribosomes to be positioned at the start codon, we tested whether leaderless ORFs were enriched in shorter reads compared to ORFs preceded by a short leader. Leaderless ORFs, such as *MT-CO2*, *MT-CO3*, *MT-ND4L* contained MRPFs mainly 20-23 nucleotides in length at the 5' end (Figure 4C, top). *MT-ATP8*, which contains a start codon beginning at the 3rd nt, contained footprints were 22-24 nt long and *MT-CO1,* with a start codon at 4th nucleotide, contained MRPFs 23-24 nucleotides long (Figure 4C, middle). Not only did we observe longer MRPFs in short leader ORFs, the size of the MRPFs also appeared proportional to the location of the start codon. Together, these results indicate that the size of the footprint is directly related to the start codon position at the 5' end of mitochondrial mRNAs.

Two genes, *MT-ND*1 and *MT-ND5,* deviated from this trend. The annotated start site for *MT-ND5* is a leaderless AUA codon (a start codon in mitochondria) but we observed few 20-22 nt MRPFs (Figure 4C, bottom). Similarly, the annotated start site for *MT-ND1* is also an AUA codon which begins at the 3rd nt and it also lacks reads for MRPFs that are smaller than 25 nts. Instead, MRPFs derived from the 5' end of both genes are enriched in longer reads: 27 nts long for *MT-ND5* and 28-29 nts long for *MT-ND1*. Curiously, the third codon for both genes is an in-

frame AUG and the read lengths proportionally correspond to mitoribosomes positioned at these start codons, suggesting mitoribosomes presumably initiating translation at these sites. Using the protein mass spectrometry, we tested whether peptides matching the N-terminus of these proteins would correspond to initiation at the canonical start codons or alternative start codons. We identified a peptide that mapped to specifically the truncated version of MT-ND1 and not the full-length peptide, indicating that translation can initiate from the alternative start codon (Figure 4D). While we did not identify N-terminal peptides mapping to MT-ND5 from our mass spectrometry data, the protein mass spectrometry database MassIVE[27] covered the N-terminal region of MT-ND5 (Figure 4E). Consistent with our mitoribosome profiling data, the majority of N-terminal fragments did not include the amino acids from the annotated start or second codon but began with methionine at codon 3. We also observed that MRPFs on *MT-ND6,* which initiates at the first of two AUGs codon immediately at the 5' end, correlated to ribosomes positioned at both AUG codons, indicated that this gene may initiation translation from the second AUG codon as well. However, we did not observe any peptides mapping to the N-terminus of *MT-ND6*. Together, this data shows that *MT-ND1* and *MT-ND5* may not initiate from their canonical AUA codons but may use alternative in-frame AUG codons. Additionally, our findings indicate that the mapping of mitochondrial translation initiation events by size, rather than 5' offset is an accurate strategy.

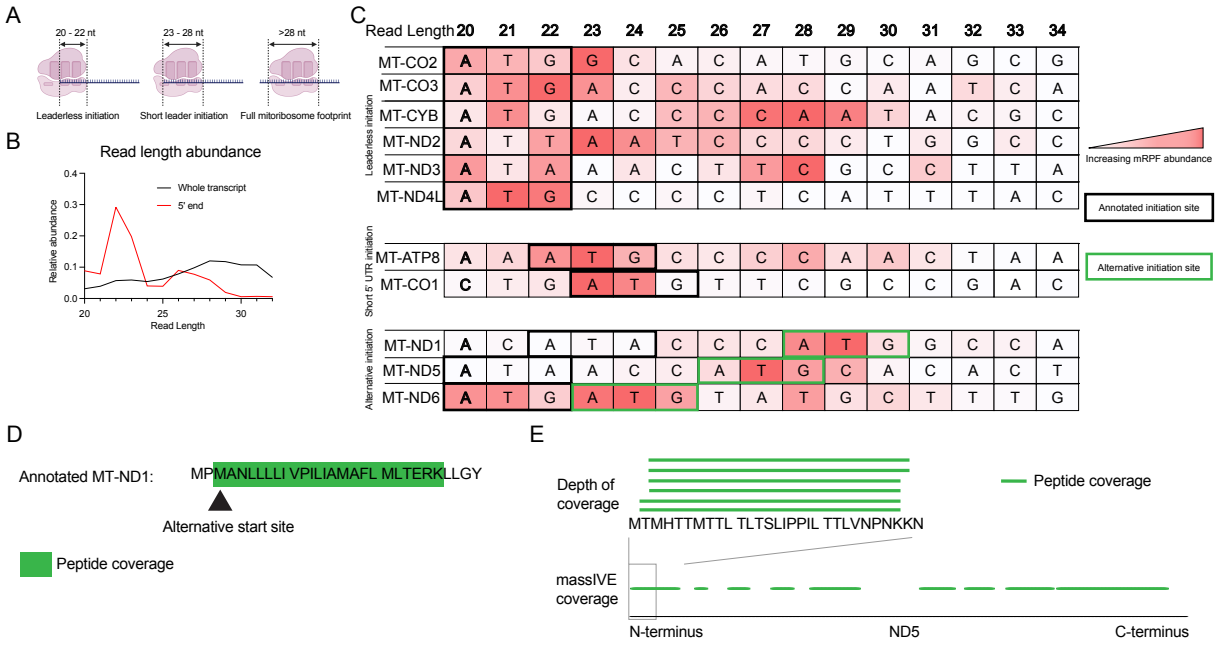

Figure 4. MRPF sizes indicate use of alternative initiation sites.

(A) Schematic representation of changing MRPF sizes as a function of the mitoribosome's position near the 3' end of mRNAs. (B) Read length measurement of reads mapping to the 5' ends of mitochondrial transcripts (red) compared to the entire ORF (black). (C) Read lengths of MRPFs mapping to the 5' ends of mitochondrial transcripts. Each row contains the nucleotide sequence of each transcript starting from the 5' end. The coloring of the box is proportional abundance of MRPFs with read lengths from 20-34 nts. Nucleotide sequence and read abundance are overlayed to compare the abundance of reads to potential ribosome start sites. Transcripts are grouped by being leaderless, having a leader, or having a potential alternative start site. Black boxes indicate annotated start codons and green boxes indicate alternative initiation sites. (D) Visual representation of the peptide coverage (green bars or highlights) from mass spectrometry data covering the N-terminus of *MT-ND1* or (E) *MT-ND5.*

Mitoribosome profiling reveals novel translation events

During our analysis of 5' end mapping we did not remove reads mapping to ncRNAs prior to mapping to the mitochondrial genome and performing P-site assignment. Consequently, we observed that some tRNAs and rRNAs had an abundance of reads mapping to 13 nts away from their 5' ends, similarly to what we observe on mRNAs for translation initiation. In addition, some of these ncRNAs contained a potential start codon near the 5' end. These reads could derive from either random digestion of the abundant background of ncRNAs or be genuine MRPFs. We hypothesized that if these reads were the result of mitoribosomes initiating or attempting to initiate translation at the 5' end of the ncRNAs, then we could use the same analysis of read length distribution at 5' ends of mRNAs we performed above and determine of the sizes correlated to an AUG, AUU, GUG, or UUG as potential start codons. While GUG and UUG are not used in canonical mitochondrial initiation they were included in the search because noncanonical initiation has been observed at GUG[29] and both are frequently utilized in bacterial initiation. We identified sites on 4 tRNAs that contained MRPFs with a size that correlated to either AUG or UUG codons (Figure 5A). The predicted ORFs and thus resulting potential translation events vary widely. On *MT-TF*, the start codon is followed by an immediate stop codon. The ORF on *MT-TI* is 3 codons long and terminates with the non-canonical mitochondrial termination codon AGA. On *MT-TY, a* 7-codon ORF is terminated by a UAA codon. *MT-TS1* contains the largest open reading frame identified on tRNAs, at 20 codons and it terminates with at the U AGA sequence, as used in *MT-CO1*[30]. Additionally, we identified a putative start site at the 5' end of *MT-RNR1* (Figure 5A). However, this site did not occur at a canonical AUG or AUU codon, but instead at a UUG codon. This putative start codon is followed by a 58-codon open reading frame and terminates at a UAG stop codon.

To determine whether translation elongation occurs at these putative ORFs, we determined the read phasing characteristic for MRPFs derived from translating ribosomes at these open reading frames. However, the sites on *MT-TF*, *MT-TI*, and *MT-TY* are short and close to the 5' end, hindering phasing analysis and thus do not allow us to conclude whether our sequence reads are indicative of translation. While the ORF on *MT-TS1* is longer, MRPF density was low and we did not observe read phasing and thus is likely not translated (not shown). The reads mapping to the ORF on *MT-RNR1* had a phasing profile similar to those mapping to the 13 canonical mitochondrial genes (Figure 5B). In contrast, reads mapping to the remainder of *MT-RNR1* or mapping to the region of *MT*-RNR2 that codes for the putative mitochondrial micropeptide Humanin, shows no or conflicting read phasing. When compared to the amino acid

composition of the mitochondrially-encoded proteome, the amino acid composition of the resulting peptide shows a stronger positive correlation (R^2=0.534) (Figure 5C) to the remaining non-coding portion of *MT-RNR1* (R^2=0.241) (Figure 5D). Although mitoribosome profiling suggests that this putative ORF is translated, we did not detect any corresponding peptides in the protein mass spectrometry of isolated mitochondria. However, coverage of these peptides from our mass spectrometry dataset as well as the MASSIVE database does not cover many regions of the mitochondrial proteome and the absence of a spectra is not necessarily evidence the peptide is not translated. Despite not finding a peptide, read phasing analysis suggests this region of *MT-RNR1* is translated and could encode a 15th mitochondrial protein. Overall, this data shows that our mitoribosome profiling can be used as a tool to study non-canonical translation events resulting from ncRNAs.

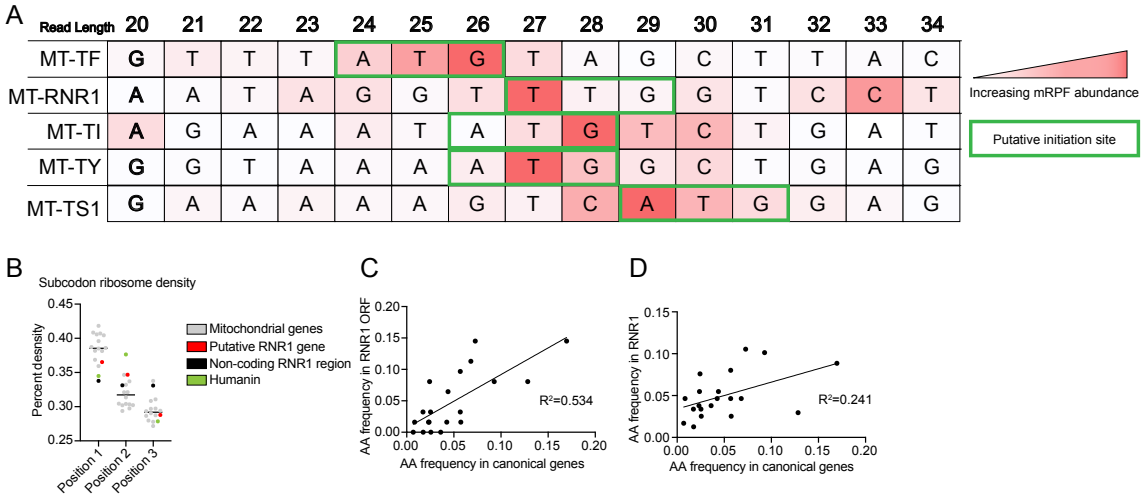

Figure 5. MRPFs on ncRNAs indicate potential novel ORFs

(A) Read lengths of MRPFs mapping to the 5' end of ncRNAs. Each row contains the nucleotide sequence beginning from the 5' end of listed ncRNAs. The coloring of the box is proportional the abundance of MRPFs with read lengths 20-34 nts. The nucleotide sequence and read MRPF abundance are overlayed to identify potential new start sites. Putative start sites are indicated by green boxes. (B) Read phasing analysis of all canonical ORFs (grey), a mutative ORF located on *MT-RNR1,* the non-coding region of *MT-RNR1,* and Humanin. (C) A correlation plot comparing the frequence of amino acid usage in canonical ORFs compared to the putative ORF on *MT-RNR1* or (D) a non-coding region of *MT-RNR1.*

Discussion

In this work, we sought to understand the mechanisms of bacterial protein synthesis inhibitors on their primary off-target, the mitoribosome. We found that inhibitors of bacterial translation initiation, retapamulin, tiamulin, and josamycin, also act as initiation inhibitors in mitochondria. Likewise, chloramphenicol and linezolid selectively inhibit elongation when the mitoribosome carries either an alanine, serine, or threonine in the penultimate position of the nascent peptide, matching their mechanism in bacteria. In contrast, the context-dependence of telithromycin differed when arresting mitoribosomes, wherein it preferentially arrested translation at R/K/A-X-K motif.

Context-dependent translation arrest by chloramphenicol and linezolid suggests similarities between binding of the bacterial ribosome and the mitoribosome. Structural and biochemical studies have demonstrated that binding of chloramphenicol and linezolid is stabilized by alanine, serine, or threonine in the penultimate position of nascent peptide[31,32]. Our findings suggest a similar or identical interaction is occurring in the mitoribosome. In contrast, these inhibitors are unable to inhibit peptide bond formation in bacteria when glycine is positioned in the A-site, due to the minimal size of the side chain, allowing the inhibitor and the aminoacyl-moiety to coexist in the PTC. We did not observe a lack of mitoribosome translation arrest when glycine was positioned in the A-site. This may be caused by an insufficient depth in sequencing, that the relatively small mitochondrial genome limits the variety of stalling and non-stalling events we can observe, or that chloramphenicol and linezolid are capable of arresting translation when glycine is in the A-site. Recently, mitoribosome profiling analysis showed that chloramphenicol added during lysis did not induce context-dependent translation arrest[33]. This suggests that during lysis, there is insufficient mitochondrial translation to induce a redistribution of mitoribosomes rather than an inability of the chloramphenicol to induce context-dependent arrest.

During preparation of this manuscript, two other groups studied the effect of translation inhibitors on mitochondrial translation. One group's mitoribosome profiling in the presence of chloramphenicol and linezolid agreed with our observed context-dependent translation arrest[34]. Their structure of linezolid in the mitochondrial peptidyl-transferase center also suggested some

nuanced differences in binding from the bacterial ribosome. Another group carried out mitoribosome profiling in the presence of retapamulin[33]. However, they observed additional ribosome density throughout the coding region and potentially some associated with putative start codons. This differs significantly from our observed accumulation at only annotated start sites and may be due to a differences in mitoribosome profiling methods. Future work may reveal how each strategy contributes to our understanding of mitochondrial translation.

Inhibition of mitochondrial translation through either small molecules or mutations localized in mitochondrial translation factors can induce a wide range of pathologies[35-37]. However, it is unclear how or whether the relationships between the mechanism of translation inhibition relates to the resulting disease. Recently the proteins mtRF-R and MTRES1 were shown to act as quality control factors, rescuing stalled mitoribosomes by removing the nascent peptide and peptidyl-tRNA[38]. As the mitoribosome can arrest translation through a variety of mechanisms and small molecules, it remains unclear whether these factors can rescue all stalled mitoribsomes. Further work to dissect the relationship between mechanisms of inhibition, activation of rescue factors, and stress response can complement our understanding of pathologies resulting from mitoribosomopathies and antibiotic treatment.

In this study, we also reported that the translation start codon of two mitochondrial genes, *MT-ND1* and *MT-ND5*, do not use the annotated AUA codon, but instead begin translation from an nearby in-frame AUG codon. Others have noted that haplogroups L1b and F2 contain a single nucleotide polymorphism at either position T3308C or T12338C respectively, which disrupts the annotated AUA start codons of *MT-ND1* and *MT-ND5* yet do result in any discernable pathogenicity[39-41]. Authors noted that the same AUG codons we identified as start codons could be used in lieu of the canonical AUA start codons. Because we observe MRPF lengths primarily correlating with the AUG codons, we suggest that these downstream start codons are the primary start site for these genes, though this does not preclude the use of both sites. Given the limited examples of translation initiation sites inherent to the mitochondrial genome, the reannotation of 2 sites is a significant revision and can guide future work to understand how start codon selection occurs in mitochondria.

Recently, mitoribosome profiling was used to identify an open reading frame in region immediately 3' of the gene *MT-ND5,* which we confirmed observing in our profiling experiments[42]. However, this uses an internal initiation mechanism for polycistronic genes, similar to *MT-ND4* and *MT-ATP6.* Until now, accurate mapping of mitochondrial translation events at the 5' ends of transcripts has been unavailable. We identified several putative translation initiation events

occurring on mitochondrial MT-tRNAs and *MT-RNR1*, based on the size of the reads occurring at the 5' end of the RNA and their relationship to a start codon localized in the sequence. The site of *MT-RNR1* was further supported by analysis of the resulting open reading frame, suggesting translation initiation and elongation occurs. Curiously, $tRNA^{Tyr}$, encoded by *MT-TY*, is polyadenylated during processing, which may be related to our identification of a ribosome binding event on this tRNA[43]. Due to both the short ORFs, minimal read coverage, and lack of a detectable peptide we could not determine if translation elongation occurred on the mitochondrial tRNAs. Future work is needed to understand whether these are actual translation events and to determine the function of these potential novel peptides. Together our findings improve our understanding of mitochondrial translation initiation, gene encoding, and the mechanisms of translation inhibition.

Materials and Methods

Isolation of mitoribosomes for profiling and western blotting

Mitoribosome profiling was carried out in HEK293 cells. 15 cm plates were treated with either DMSO as a control or 100 µg/mL of protein synthesis inhibitor for 1 hour, washed with 4°C PBS, and flash frozen by floating the plate on liquid nitrogen. Cells were collected by scrapping on ice with 600 µL of lysis buffer (1.5x Lysis buffer (30 mM Tris–HCl pH 7.8, 150 mM KCl, 15 mM $MgCl_2$, 1.5 mM DTT, 1.5% Triton X-100, 0.15% NP40, 1 × complete phosphatase and protease inhibitors)) and lysed by passing through a 27-G needle 10 times. Lysate was centrifuged for 10 minutes at 21,000 g at 4°C and the supernatent was collected. 300 µL of lysate was footprinted with either digested with 1,5000 U of MNase, 10 µL of SUPERase-In, and 5 mM of $CaCl_2$ at 22°C for 1 hour and stopped by adding EGTA to a concentration of 6 mM or with 1 U/µL of RNase I at 22°C for 1 hour and stopped by adding 10 µL of SUPERase-In. The digestion was clarified by centrifugation at 21,000 g for 5 minutes. Lysates were loaded onto a 5%-30% sucrose gradient (20 mM Tris-HCl ph 7.8, 100 mM KCl, 10mM $MgCl_2$, 1 mM DTT) and spun for 2.5 hours at 40,000 rpm in a SW40 rotor. 55S mitoribosome fractions were collected using a BioComp Fractionator and MRPFs were isolated by SDS/hot phenol/chloroform extraction.

Mitoribosome profiling library construction

Samples were run on a 15% TBE-Urea polyacrylamide gel and footprint sizes from 15-34 nts were excised and footprints were eluted in 0.3 M NaOAc, 1 mM EDTA, and 0.25% SDS followed by purification by Oligo Clean & Concentrator. For MNase, RNase I, Retapamulin, Tiamulin, and their untreated control, samples were prepared as previously described[44]. Footprints were treated with PNK, followed by ligation to a preadenylated linker (Phos/NNNNN-Barcode-AGATCGGAAGAGCACACGTCTGAA/3DDC/) using T4 truncated RNA ligase 2 (K227Q). Samples were then treated with deadenylase and RecJ exonuclease to remove unligated linkers. Samples were pooled, purified using an Oligo Clean and Concentrator kit. Ribosomal RNAs were depleted using QIAseq FastSelect kit. Pooled cDNAs were generated by reverse transcription with Superscript 3 and a reverse transcription primer (5Phos/NNAGATCGGAAGAGCGTCGTGTAGGGAAAGAG/iSp18/GTGACTGGAGTTCAGACG TGTGCTC). cDNAs were run on a 10% TBE-Urea gel and size selected. cDNAs were extracted from cut gel pieces with a DNA gel extraction buffer (0.3 M NaCl, 1 mM EDTA, 10 mM Tris pH 8) shaking overnight at 700 rpm at 20°C, followed by ethanol precipitation and resuspension in 10

mM Tris pH 8. cDNAs were circularized using CircLigase ssDNA Ligase. Libraries were amplified using Phusion DNA Polymerase, size selected on an 8% native gel, eluted using DNA gel elution buffer, and resuspended in 10 mM Tris pH 8.

In the case of all other ribosome profiling experiments, samples were treated with Quick CIP. 3' adapters (5'-rAppNN-UMI-TGGAATTCTCGGGTGCCAAGG-L) were ligated using Rnl2(1-249)K227Q ligase for 2 hours at 16°C and pooled. Pooled samples were treated with T4 PNK for 30 minutes at 37°C. A 5' chimeric DNA-RNA adapter (GTTCAGAGTTCTACAGTCCGACGATCrNrNrNrN) was ligated to the samples with T4 RNL1. Reverse transcription was carried out using SuperScript IV followed by PCR amplification by Taq DNA polymerase. Libraries were size selected using a 3% agarose Pippin prep and final libraries were PCR amplified. Samples were sequenced as either singe- or paired-end 50 nt reads on either a nextseq550 or nextseq2000.

Mitoribosome profiling analysis

Adapter sequences were removed with cutadapt and aligned with bowtie2 to a fasta containing rRNA and tRNA sequences. To identify stalling sites, reads were mapped to a custom mitochondrial transcriptome containing all 5' UTRs, coding sequences, and 3' UTRs for all processed mRNAs. Reads were aligned with bowtie2. A 13 nt P-site offset was assigned using Plastid and mapping reads relative to the start codons of *ND4*, *ATP6*, *MT-ND5-dORF.* We determined the relative ribosome density by normalizing the number of reads at each codon by the total number of reads mapping to its respective gene for each sample. Fold change was then calculated by dividing the relative ribosome density in a treated sample to an untreated sample. To identify putative novel translation initiation sites we mapped our footprints to the entire mitochondrial genome and visually inspected the 3' ends of mitochondrial tRNAs and rRNAs.

Mass spectrometry analysis of mitochondrial proteins

Mitochondria were isolate from $2x10^7$ HEK293 cells using the Mitochondria Isolation Kit for Cultured Cells. Mitochondrial proteins were processed for trypsin/LysC digestion and the digested peptide mixture was then concentrated and desalted using C18 column. Reconstituted desalted peptides in 25 µl of 0.1% formic acid. 12 µl of peptides from each sample was analyzed by 110 min LC/MS/MS run. The LC/MS/MS analysis of tryptic peptides for each sample was performed

sequentially with a blank run between each two sample runs using a Thermo Scientific Orbitrap Exploris 240 Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Peptides from trypsin digestion were loaded onto a peptide trap cartridge at a flow rate of 5 µL/min. The trapped peptides were eluted onto a reversed-phase Easy-Spray Column PepMap RSLC, C18, 2 µM, 100A, 75 µm × 250 mm (Thermo Scientific) using a linear gradient of acetonitrile (3-36%) in 0.1% formic acid. The elution duration was 60 min at a flow rate of 0.3 µl/min. Eluted peptides from the Easy-Spray column were ionized and sprayed into the mass spectrometer, using a Nano Easy- Spray Ion Source (Thermo) under the following settings: spray voltage, 1.6 kV, Capillary temperature, 275°C. Other settings were empirically determined. Raw data files were searched against human protein sequences database and client target protein sequences database using the Proteome Discoverer 2.5 software (Thermo, San Jose, CA) based on the SEQUEST algorithm. Carbamidomethylation (+57.021 Da) of cysteines was set as fixed modification, and Oxidation / +15.995 Da (M), Phospho / +79.966 Da (S, T, Y), and Deamidated / +0.984 Da (N, Q) were set as dynamic modifications. The minimum peptide length was specified to be five amino acids. The precursor mass tolerance was set to 15 ppm, whereas fragment mass tolerance was set to 0.05 Da. The maximum false peptide discovery rate was specified as 0.01. The resulting Proteome Discoverer Report contains all assembled proteins with peptides sequences and peptide spectrum match counts (PSM#) and MS1 peak area relative abundance.

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