# Exploring the importance of translational regulation in the inflammatory responses by a genome-wide approach

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## ABSTRACT

*It is widely recognized that exercise has an important role in inflammation regulation. To understand inflammatory mechanisms, extensive studies on the transcriptome and proteome have been conducted. However, interpreting these results is difficult, partly due to technical difficulties that impose some restriction on the accuracy and comprehensiveness of measurements. Here we first mention some limitations of studies involving large scale proteomics and high-throughput transcriptomics and further introduce a newly developed genome-wide translational analysis which may overcome some of the limitations and discover novel cellular dynamics. We then show preliminary results obtained by conducting a genome-wide translational analysis of the early inflammatory response of macrophages in response to lipopolysaccharide (LPS), and discuss the potential to identify novel factors by employing a genome-wide translational analysis.*

**Key words:** translational regulation, translational isoform, up stream open reading frame (uORF), inflammation, macrophages, gene expression

## INTRODUCTION

Exercise has numerous benefits for health and physical fitness, and in particular has proven effective in the management of numerous disease states associated with chronic inflammation, including obesity and diabetes. While research has investigated the anti-inflammatory effects of exercise, the complicated mechanisms are yet to be fully understood. In an attempt to understand the anti-inflammatory effect of exercise, and to reveal the underlying molecular mechanisms,

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genome-wide analyses such as microarray, high-throughput mRNA sequencing (mRNA-Seq), and proteomics are promising tools.

**Limitation in large-scale proteomics**

While large-scale proteomics techniques may provide further insight into molecular mechanisms there are some limitations to consider. One limitation is the underestimation of low-abundance proteins (19, 31). In mass spectrometry-based proteomics, liquid chromatography and/or two-dimensional gel electrophoresis are typically employed to separate proteins at a high-resolution level. On a two-dimensional gel, the appearance of a low-abundance protein can be masked by other high-abundance proteins (19, 31). In the case of proteins associated with cytokine responses, many physiologically meaningful proteins, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α, are produced at very low concentrations. Concentrations of other abundant proteins, such as $\alpha_2$-macroglobulin and serum albumin, may be up to 12 orders of magnitude higher than those of cytokines (3, 6, 20). Although the sensitivity to detect less abundant proteins can be improved by using an immunoaffinity column to remove highly abundant proteins, efficiency of removal is only 80 to 90 %, meaning that there is still a large excess of $10^{11}$ proteins (7, 20). In addition, proteins that interact with the target proteins to be removed may also be filtered out, leading to an unfavorable sample bias (7). These issues may lower the possibility to identify less abundant proteins in a large-scale proteomics.

**Limitation in microarray/mRNA-Seq**

Detection of low-abundance proteins can be achieved by the use of microarray and mRNA-Seq. However, one concern is that these techniques do not account for post-transcriptional modification (11). Many studies compared protein levels and microarray/mRNA-Seq data and showed low correlations between them ($R^2$ = 0.17 - 0.41), suggesting that the mRNA level is not always sufficient to estimate its protein abundance (8, 10, 18, 28, 29). One study further revealed that this low correlation is mainly due to the ignorance of translational events, reporting a correlation coefficient of $R^2$ = 0.41 between mRNA and protein levels whereas

**Table 1.** Advantages and disadvantages of large-scale proteomics, microarray/mRNA-Seq, and ribosome profiling.

| | Advantages | Disadvantages |
|---|---|---|
| **Large-scale proteomics** | - Direct quantification | - Underestimation of low-abundance proteins |
| **Microarray / mRNA-Seq** | - Genome-wide quantification including low-abundance factors | - Low correlation between mRNA and protein abundance |
| **Ribosome profiling** | - Genome-wide quantification including low-abundance factors<br>- Better estimate of protein abundance<br>- Able to identify translational dynamics | - Substantial amount of rRNA and sequence read loss |

measured translation rates and protein abundance were very strongly correlated ($R^2 = 0.95$) (22). This finding indicates that translation rates certainly exert significant influence on protein abundance and that post-translation has only a nominal effect (22). Given the above, it is evident that the translation rate regulated by post-transcriptional control is a critical determinant of protein abundance.

**Advantages in ribosome profiling**

A recently developed strategy, named ribosome profiling, provides a robust measurement of translational profile. Ribosome profiling was first innovated by Ingolia et al., in 2009 (11). This method achieves a technical breakthrough by deep-sequencing ribosome-protected mRNA fragments (RPF) and quantitating ribosome density on mRNA (11). This mRNA-Seq-based ribosome profiling (or RPF sequencing) provides a powerful genome-wide approach that is reproducible and comprehensive qualities which are lacking in large-scale proteomics. The direct measurement of RPF is able to achieve a better estimate of protein abundance and also monitor translational dynamics such as frame-shifted protein synthesis, translational isoform of proteins, and the alteration of translation-dependent protein synthesis without changing mRNA levels (11, 12, 17). Thereby, it is likely that some unrevealed factors attributable to translational regulation can be discovered and that they may have roles in the anti-inflammatory effects of exercise. Here we introduce ribosome profiling (originally from Ingolia et al. (11)) and present preliminary data obtained using this method to investigate the early inflammatory response in macrophages exposed to lipopolysaccharide (LPS).

## METHODS

The simplified workflow of ribosome profiling modified for this study is as follows (Fig. 1): First, cultured cells are incubated with cycloheximide (Sigma, St. Louis, MO) to stall translating ribosomal complex on the mRNA. Total RNA is then extracted and digested by RNase If. Because mRNA fragments encompassed by the ribosome are not digested, they are further purified as monosome by sucrose cushion. Gel purification is then carried out to extract RPF. As RNase If digestion dephosphorylates the 5' end and phosphorylates the 3' end, both ends are phosphorylated and dephosphorylated, respectively. Sequence library preparation for RPF is then conducted and sequenced by a next generation sequencer.

**Total RNA extraction for mRNA-Seq and RPF sequencing**

RAW 264 macrophages (DS Pharma Biomedical, Osaka, Japan) were cultured at a concentration of 2.5 x $10^5$ cells/ml in media (DMEM, 2 mM Glutamine, 10% FBS, 100 units penicillin and 100 μg/mL streptomycin) for 24 hours and then harvested. Ensuring cells were approximately 80 ~ 90% confluency, 100 ng/ml LPS (*E. coli* O55:B5; Sigma) or vehicle (DMEM) was added into the media and incubated for 30 min. To stall ribosomal complex on mRNA, cycloheximide (final concentration of 100 μg/ml) was added and cells were incubated for 5 min, followed by washing with PBS (including 100 μg/ml cycloheximide). Cells were lysed in 400 μl lysis buffer (20 mM TrisHCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml cycloheximide, TURBO DNase I 25 U/ml

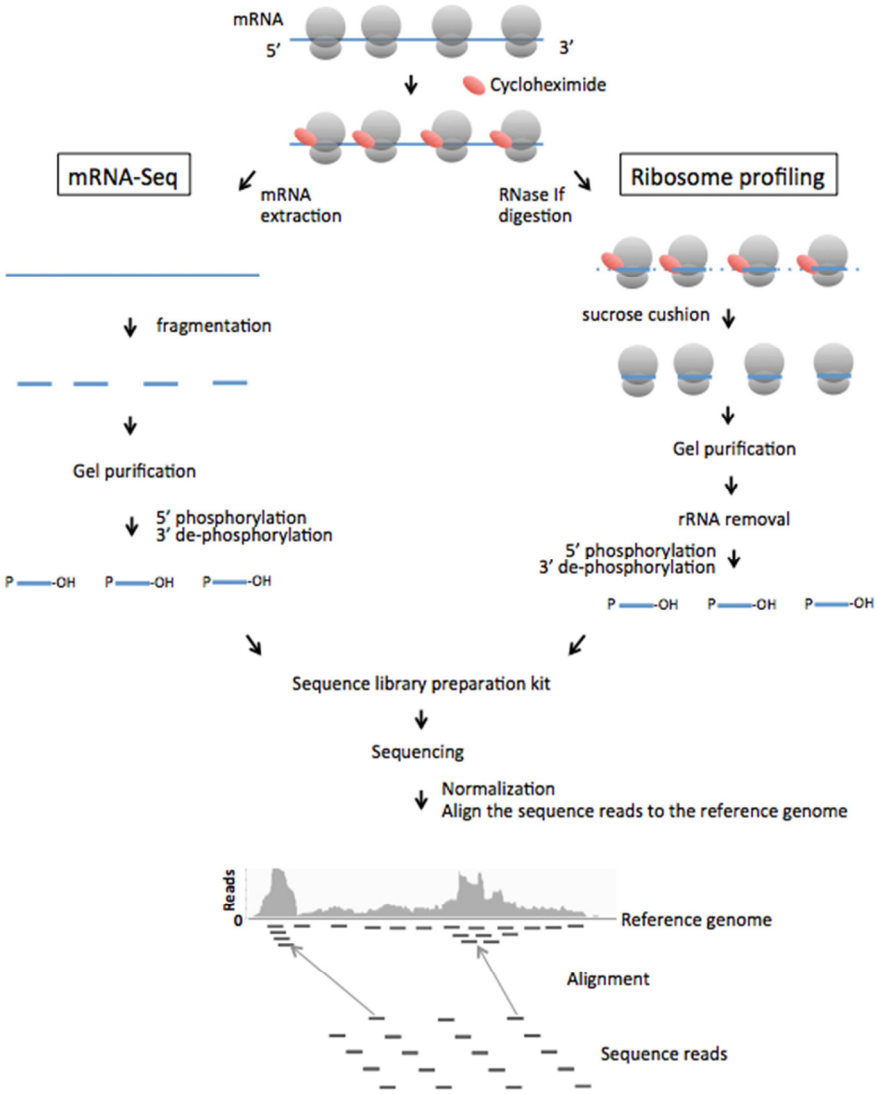


**Fig. 1.** The concept of ribosome profiling
The simplified workflow of ribosome profiling is shown.

(Ambion, Life Technologies, Carlsbad, CA), 1% Triton X-100 (Sigma)), followed by trituration through a 26-G needle. After centrifugation at 20,000 *g* for 10 min at 4°C, the supernatant was collected.

**RNA fragmentation for mRNA-Seq**

After purification of total RNA by miRNeasy Mini Kit (Qiagen, Hilden, Ger-

many), poly(A) RNA extraction was carried out by Dynabeads mRNA DIRECT Micro Kit (Ambion). For fragmentation, 2x alkaline buffer (90 mM $NaHCO_3$, 10 mM $Na_2CO_3$, 2 mM EDTA) was mixed with mRNA and incubated at 80°C for 15 min. The reaction was stopped by adding an ice-cold solution (1.5 µl GlycoBlue (Ambion), 10 µl 3M NaOAc, 48.5 µl RNase-free water) and 150 µl isopropanol, followed by standard precipitation protocols.

**Ribosome protected mRNA fragment (RPF) purification**

To first digest mRNA which was not protected by the ribosomal complex, 15 µl RNase If (New England Biolabs, Beverly, MA) was added to the total RNA lysate and incubated for 45 min at room temperature. The reaction was stopped by adding 10 µl SUPERase· In (Ambion). While digesting, sucrose cushion buffer (34% sucrose, 20 mM TrisHCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µg/ml cycloheximide, 20 U/ml SUPERase· In) was prepared and loaded into a polycarbonate tube (Beckman Coulter, Palo Alto, CA). The digested sample was then loaded onto the sucrose cushion at a ratio of 1:3. After centrifugation for 4 hours at 400,000 *g*, 4°C in a MLA130 rotor (Beckman Coulter), the pellet was purified by miRNeasy Mini Kit, followed by precipitation as described previously.

**Size selection of RPF and fragmented mRNA**

The mixture of 2x Novex TBE-Urea sample buffer (Invitrogen, Life Technologies, Carlsbad, CA) and each purified RNA sample, small RNA II Marker (Funakoshi, Tokyo, Japan), and synthesized oligo markers (Greiner, Tokyo, Japan, 32 nucleotide (nt) upper marker: 5'-AUGUACACGGAGUCGAGCUCAACCCGCAACGA, 26nt lower marker: 3'-AUGUACACGGAGUCGACCCGCAACGA, both 3' ends were phosphorylated and both 5' ends were dephosphorylated) was heat-denatured at 70°C for 3 min. Samples were then loaded on a denaturing 15% polyacrylamide TBE-urea gel (Invitrogen) and run for 65 min. The gel was stained with SYBR Gold (Invitrogen) for 5 min. Specific regions (26 ~ 32nt for RPF and 25 ~ 45nt for mRNA-Seq) were excised and disrupted, followed by incubation in 360 µl RNase-free water at 70°C for 10 min. All gel and liquid were transferred into a Spin-X column (Corning, NY), followed by centrifugation at 20,000 *g* for 2 min. Precipitation was carried out as described previously.

**rRNA deletion, 3'dephosphorylation, and 5' phosphorylation**

Because RPF samples contain a significant amount of ribosomal RNA (rRNA), rRNA removal was conducted using RiboMinus (Invitrogen). After rRNA deletion, the total amount of RPF was reduced to approximately 1/200. To prepare samples for adaptor ligations, both samples (RPF and randomly fragmented mRNA) were 3'-dephosphorylated and 5'-phosphorylated. Dephosphorylation was first carried out at 37°C for 1 hour in a reaction mixture (T4 polynucleotide kinase buffer without ATP, 10 U/µl T4 polynucleotide kinase (New England Biolabs), 20 U/µl SUPERase· In), followed by the addition of 1 mM ATP (New England Biolabs) and immediate 5' phosphorylation for 30 min. Precipitation was then carried out to concentrate samples for sequence library preparation.

**Sequence library preparation**
Ion Total RNA-Seq Kit v2 (Ion Torrent, Life Technologies, Carlsbad, CA) was used to prepare the sequence library according to the manufacturer's instructions. However, because of the difficulty of differentiating target fragments and primer dimmers mainly coming from reverse transcription PCR, gel purification was conducted to purify a target product. After running the gel, the target region, around 63nt (fragmented sample size: 30nt and ligated adaptors: 33nt), was excised, followed by RNA purification from the gel as described previously and isopropanol precipitation.

**Sequencing**
For emulsion PCR, the sequence library was processed by Ion PGM Template OT2 200 Kit (Ion Torrent). Sequencing was then carried out by Ion PGM sequencer (Ion Torrent), Ion PGM Sequencing 200 Kit v2 (Ion Torrent) and Ion 318 Chip Kit v2 (Ion Torrent) according to the manufacturer's protocols. Sequenced reads were mapped to reference genomes (mm10 and Coding DNA sequence (CDS) created from RefSeq mRNA). RefSeq mRNA (http://www.ncbi.nlm.nih.gov/refseq/) and mm10 (http://genome.ucsc.edu) genome was obtained. CDS was created by the use of Biomart-MartView (http://www.biomart.org/biomart/martview) (13). As for the reads mapped to multiple locations, the best hit read was selected. Sequenced reads aligned to mm10 were visualized by Integrative Genome Viewer (27).

**Data analysis**
Sequenced reads aligned to CDS were normalized by CDS length and library size (Reads Per Million per Kilobase of CDS (RPKM)). Unique genes ≥ 15 RPKM in at least one of the four conditions (RPF sequencing without LPS, RPF sequencing LPS 30 min, mRNA-Seq without LPS, mRNA-Seq LPS 30 min) were further analyzed. Pair-wise t tests were used to compare read ratios in 3nt periodicity and *P* values were adjusted using a Bonferroni correction. Pearson's correlation coefficients were also calculated.

## RESULTS AND DISCUSSION

Extensive studies have been carried out using proteomics and transcriptomics. In addition to these two major omics, the use of translational profile is now gaining attention. Ribosome profiling, sequencing RPF, now makes it possible to conduct robust measurements of translational dynamics and may reveal novel perspectives of cellular regulation. We conducted a genome-wide translational analysis by ribosome profiling. Our preliminary data suggest the potential to discover novel factors that may have critical roles in regulating inflammatory and anti-inflammatory responses to exercise.

**Genome-wide detection of translational dynamics**
Large-scale proteomics is limited by its lack of genome-wide detection and reproducibility of results. In the present study, however, mRNA-Seq-based RPF sequencing was reproducible. A very strong correlation coefficient, $R^2 = 0.96$,

was obtained from the two complete biological replicates (both LPS stimulated) including genes with low translation (≥ 15 RPKM). Although there are some sequencing biases depending on genome sequence, these biases are also highly reproducible. These biases were well conserved over two completely different sequencing runs (samples from LPS stimulated and without LPS) (Fig. 2), indicating that they are less likely to influence the quantification of translation between different conditions.

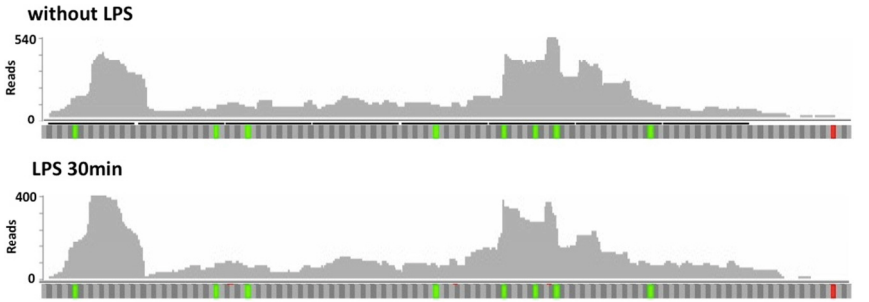


**Fig. 2.** Reproducibility of mRNA-Seq-based RPF sequencing
Reproducible bias of read coverage in a randomly selected gene in different conditions is shown. The y-axis and x-axis are read coverage and coding DNA sequence (CDS), respectively. The main CDS frame is shown below the x-axis. Light green and red squares indicate a start and stop codon, respectively. The peaks of read coverage show significant fluctuations, indicating sequence bias. However, the similar bias is also observed in a different condition, suggesting that the bias is less likely to influence the quantification of translation.

Using mRNA-Seq-based RPF sequencing more than 1500 genes were detected as moderate to highly translated genes (≥ 100 RPKM). When including those that were less translated (≥ 15 RPKM) nearly 6000 genes were detected. The number of genes detectable largely depends on two factors: sequencer performance and rRNA contamination. We used an Ion PGM sequencer designed to generate up to 10 million reads although if a sequencer capable of producing more reads (e.g., can be 200 million) is used, more genes should be quantified. As reported in previous studies, significant amounts of rRNA (40 ~ 90% of total reads) in the sequence library prevent reads from being sequenced and mapped to actual CDS (9, 11, 26). In our study, approximately half of the mapped reads (~ 48%) came from rRNA. However, we still acquired an adequate number of genes with sufficient reproducibility. Taking advantage of this robust analysis, the importance of translational regulation has been revealed in different fields, such as the mammalian cell cycle (25), nematode developmental transition (24), and maternal to zygote transition in zebrafish (14). In the field of exercise immunology it is now possible to investigate translational regulation induced during and after exercise.

In exercise, translational regulation is dynamic and complex. This is mainly because of different exercise types and the orchestrated signaling cascades of both positive and negative regulators of mammalian target of rapamycin (mTOR) signaling. mTOR signaling is known to play a key role in regulating translation

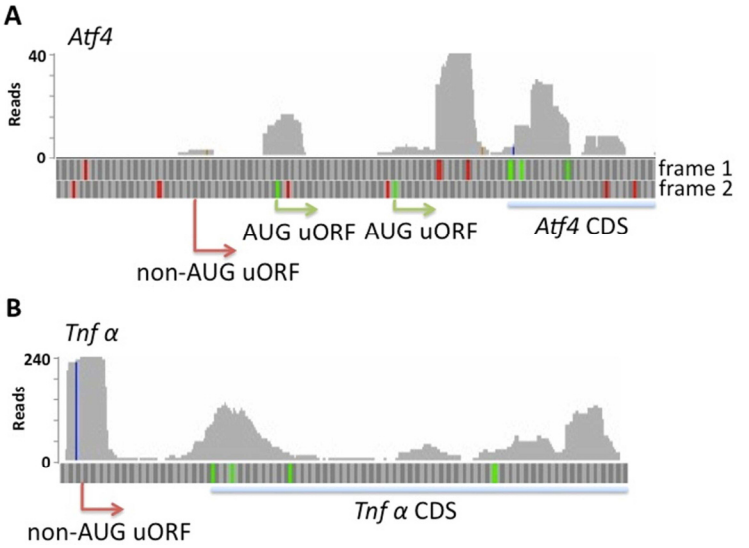


**Fig. 3.** Upstream open reading frame (uORF) in *Atf4* and *Tnf-α*
Previously identified uORF start sites in Atf4 (A) were also identified. A uORF start site in *Tnf-α* recognized in our study is shown (B). As in Fig. 2, light green and red squares indicate a start and stop codon, respectively. Frame 1 is the main *Atf4* CDS. Although it was difficult to investigate uORFs, it is now possible to monitor the translational dynamics of each uORF depending on different conditions.

by inhibiting a translational inhibitor, eukaryotic initiation factor 4E binding protein (4EBP). A recent study suggests that mTOR has some influence on more than 99% of the translation of mRNAs (26). mTOR in exercise has been well studied focusing especially on its signaling pathway. It is known that resistance exercise upregulates mTOR signaling mediated by protein kinase B (PKB) and concomitantly decreases 4EBP activation, resulting in increased protein synthesis (1). However, in the case of endurance exercise both positive and negative regulators of mTOR signaling are activated. As a positive regulator, extracellular signal-regulated kinase (ERK) 1/2, a member of the mitogen-activated protein kinase (MAPK) family, promotes mTOR signaling by inhibiting a mTOR suppressor complex (30). In turn, protein kinase A (PKA)-mediated activation of AMP-activated kinase (AMPK) shows a mTOR inhibitory effect by promoting the suppressor complex, leading to 4EBP activation (1, 2). Despite the extensive investigation of mTOR signaling cascades, its global impact on translation is still poorly understood. Ribosome profiling has the potential to capture the actual effect of both positive and negative mTOR signaling on each mRNA, and to analyze factors that are translationally activated or suppressed on a global scale.

**Other unique features detected by ribosome profiling**

In addition to genome-wide detection of translation, unique features of RPF sequencing help to identify totally new cellular regulation such as upstream open

reading frame (uORF), translational isoform of proteins, and frame-shifted protein synthesis (11, 12, 17). uORF is the translated upstream region of an annotated start codon. There are AUG-initiated and non-AUG-initiated uORFs and at least one uORF is found in approximately 50% of transcripts in mammals (16). In our study, many uORFs were recognized, including previously known uORFs, such as activating transcription factor 4 (*Atf4*) (12) and *Tnf-α* (Fig. 3). uORF is known to play a role in translational regulation. Most uORFs negatively regulate translation of the main CDS by reducing ribosomal re-initiation (4, 12, 23). For example, a uORF regulates the translation of β-site amyloid precursor protein cleaving enzyme 1 *(Bace1)*, mediating the excessive accumulation of β amyloid found in the pathology of Alzheimer's disease (32). In normal conditions, the 4th uORF of 6 *Bace1* uORFs is translated. This translation of the 4th uORF reduces the translation rate of *Bace1* CDS, consequently leading to the repression of β amyloid accumulation (32). It was difficult to monitor uORFs on a global scale. As shown in Fig. 3, however, physiologically important uORFs can be discovered by RPF sequencing and it is possible to investigate the expression and regulation of uORFs.

In addition to a suppressive effect on main CDS translation, uORFs regulate the synthesis of distinct protein isoforms. In the case of discs large (Drosophila) homolog-associated protein 3 *(Dlgap3)* (which is involved in mammalian synaptic spasticity), a uORF seems to mediate the alternative translation of *Dlgap3*, resulting in the synthesis of different *Dlgap3* isoforms from one transcript (5). These translational isoforms can be recognized by RPF sequencing. We identified a translational isoform in cluster of differentiation 14 (*Cd14*), derived from N-ter-

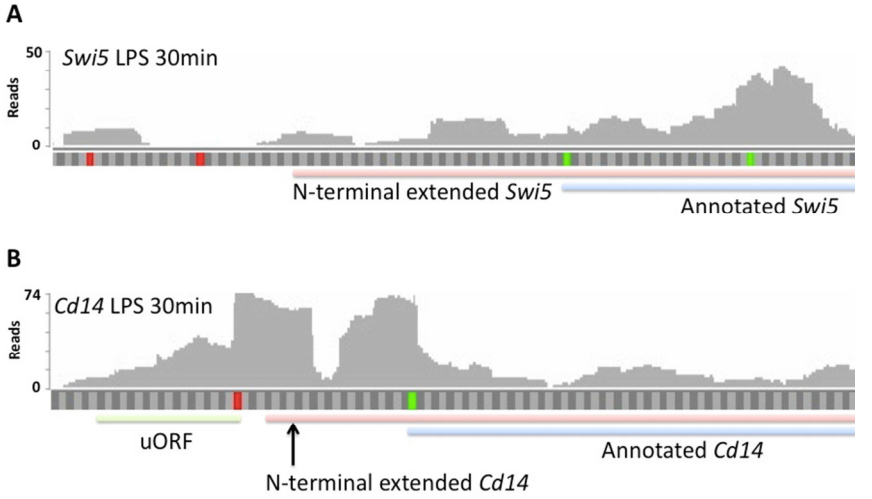


**Fig. 4.** N-terminal extensions
A previously known N-terminal extension in Swi5 recombination repair homolog (yeast) (*Swi5*) was also identified (A). A N-terminal extension in *Cd14* is shown (B). As in Fig. 2, light green and red squares indicate a start and stop codon, respectively. There was no tool available to detect translational isoforms on a global scale. However, ribosome profiling makes it possible to identify and further investigate them.

minal extension mediated by uORF (Fig. 4). The extension consists of 18 extra amino acids, with poor water solubility estimated by its iso-electric points and peptide length. As CD14 is a co-receptor of toll-like receptor 4, a receptor of LPS, this hydrophobic extension may have a role in regulating signal transition or may have an impact on its turn-over rate from the cellular membrane. As shown here, it is possible to identify translational isoforms by RPF sequencing. It may be interesting to investigate its physiological importance and the dynamics of isoform formation responding to external stimuli such as acute exercise and chronic exercise training.

Another unique feature in RPF sequencing is 3nt periodicity of aligned reads. This may make it possible to discover frame-shifted protein synthesis. A frame-shifted protein synthesis can cause a severe translational malfunction. Although a gene is expressed and the mRNA is associated with the ribosomal complex, frame-shifted translation may synthesize dysfunctional proteins. Contrary to the negative effect, it is also reported that a frame-shifted ribosome results in coding a new frame and synthesizing functional proteins. Ornithine decarboxylase (ODC) antizyme is a negative feedback regulator of ODC which is responsi-

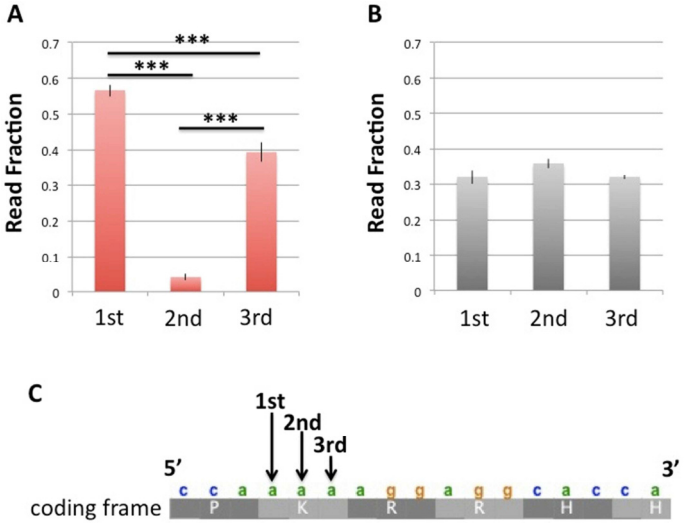


**Fig. 5.** Characteristics seen in PRF sequencing
A triplet periodicity is seen in RPF sequencing (A) but not seen in mRNA-Seq (B). 1st, 2nd, and 3rd in (A, B) indicate the 5' end of the sequenced reads mapped to 1st, 2nd, and 3rd positions of the codon in a main coding frame (C). Ribosome moves 3nt by 3nt when it translates the codon. Because RPF sequencing captures the mRNA fragments encompassed by translating ribosomes, the sequence reads show 3nt periodicity. In contrast mRNA-Seq sequences randomly fragmented mRNAs (irrelevant to ribosomal movement), and so do not show 3nt periodicity. Reads were randomly selected from the same regions of *Tnf-α* CDS for comparing RPF sequencing and mRNA-Seq. Means ± SD of two biological replicates. *** ($P < 0.01$).

ble for polyamine synthesis (21). The elevated concentration of polyamine triggers a +1 frameshift and frame-shifted protein synthesis of ODC antizyme, promoting the destabilization and degradation of ODC to regulate polyamine levels (15). A frame-shifted protein can gain a function responding to the environment. Such dynamics of frame-shifted protein synthesis can also be detected by RPF sequencing. In RPF sequencing, it is known that the 5' ends of RPF are likely to align mostly to the 1st position of a codon of a main coding frame. In contrast, the 5' ends of the reads from mRNA-Seq equally align to all the three (1st, 2nd, and 3rd) positions of a codon (11, 12). In the present study we also observed this characteristic (Fig. 5). Due to this strong periodicity, it is now possible to monitor the sophisticated dynamics of frame-shifted protein synthesis and its physiological importance (17).

## CONCLUSIONS

In this manuscript we introduce a recently developed strategy, named ribosome profiling, in which RPF is deep-sequenced. This strategy makes it possible to conduct a genome-wide translational analysis with precise and accurate measurements at a sub-codon resolution. This robust measurement may reveal novel molecular factors, including translational regulation, uORF, translational isoforms, and frame-shifted translation, which have not been discovered by large-scale proteomics, microarray, or mRNA-Seq. It will be intriguing to investigate the actual roles of these factors in the immune response to exercise.

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