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Ribosome profiling the cell cycle: lessons and challenges

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Abstract

Understanding the causes and consequences of dynamic changes in the abundance and activity of cellular components during cell division is what most cell cycle studies are about. Here we focus on control of gene expression in the cell cycle at the level of translation. The advent of deep sequencing methodologies led to technologies that quantify the levels of all mRNAs that are bound by ribosomes and engaged in translation in the cell (Ingolia et al. *Science* 324:218–223, 2009). This approach has been applied recently to synchronous cell populations to find transcripts under translational control at different cell cycle phases (Blank et al. *EMBO J* 36:487–502, 2017; Stumpf et al. *Mol Cell* 52:574–582, 2013; Tanenbaum et al. *Elife* 4:e07957, 2015). These studies revealed new biology, but they also have limitations, pointing to challenges that need to be addressed in the future.

Keywords

Anota; Babel; Translational efficiency; Elutriation

Introduction

Ever since the discovery of cyclin, a protein of periodic abundance in every cell cycle (Evans et al. 1983), the levels of hundreds of proteins have been shown to vary during the division of human (Ly et al. 2014) and budding yeast cells (de Lichtenberg et al. 2005). Regulated mRNA abundance accounts for most of the cell cycle-dependent changes in gene expression in yeast (Benanti 2016; Cho et al. 1998; Spellman et al. 1998) and human cells (Cho et al. 2001; Whitfield et al. 2002). Sampling the entire transcriptome also revealed other transcription products, such as long non-coding RNAs, that control the expression of cell cycle regulators, including the major cyclin-dependent kinase (Cdk) in budding yeast (Sole et al. 2015). Overall, changes in transcript levels explain ~90% of the variation in steady-state protein levels (Csardi et al. 2015; Li et al. 2014). The remaining variation is accounted for by regulated changes in the rates of synthesis and degradation of proteins in question (Csardi et al. 2015). Protein degradation in the cell cycle is well studied, and it has featured prominently in the control of cell cycle transitions in many organisms, starting with the first

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report of cyclin (Evans et al. 1983). On the other hand, cases of mRNAs under cell cycle-dependent translational control had been harder to come by, despite the long-recognized role of protein synthesis in cell cycle progression (Polymenis and Aramayo 2015). All that changed in the last few years, with the first reports of applying ribosome profiling in the cell cycle of human (Stumpf et al. 2013; Tanenbaum et al. 2015) and yeast cells (Blank et al. 2017). However, these new, powerful studies also revealed new challenges in the quest to identify mRNA targets of translational control in the cell cycle. Here, we summarize the distinct approaches used in each case, their findings, how they were analyzed, and experimental problems that need to be solved in future studies.

Induce or select synchronous cell populations?

Monitoring individual cells as they progress through the cell cycle can offer unprecedented resolution within diverse and undisturbed populations of cells (Di Talia et al. 2007; Kuwada et al. 2015). However, as currently implemented, analytical methods such as ribosome profiling require a significant amount of material, which limits the use of single-cell techniques in these contexts. Hence, querying cell cycle-dependent translational control events by ribosome profiling, with the current technologies available, mandates the use of synchronous cell populations. Cell synchronization methods come in two broad flavors: induction and selection synchrony (Mitchison 1971b). To induce synchronous cell divisions, some chemical, physical or genetic block in the cell cycle must first be introduced to a growing asynchronous culture. After all the cells accumulate at a specific cell cycle stage, they are typically released from the block and allowed to progress synchronously in the cell cycle. On the other hand, when a growing culture is subjected to a selection method, cells at a particular cell cycle stage are separated away and grown as a synchronous population. An inherent drawback of selection methods is the lower yield of synchronous samples, but the cell cycle is less distorted. In an unperturbed culture, growth is “balanced” because cellular components will increase by the same factor over time. In contrast, induction synchrony yields more cells, but it also inevitably leads to unbalanced growth, since arrested cells continue to grow in mass (Mitchison 1971b; Polymenis and Aramayo 2015). The above nuances of synchronization protocols are highly pertinent when translational control is in question because protein synthesis is the cardinal manifestation of cell growth (Mitchison 1971a). All these synchronization approaches were used in the ribosome profiling studies we will discuss.

The view of regulated translation in the human cell cycle: depends on how you arrest for it?

In the first study, to look for mRNAs under translational control in the cell cycle by ribosome profiling, the authors arrested HeLa cells with thymidine or with nocodazole (Stumpf et al. 2013). Exposing mammalian cells undergoing DNA replication to high concentrations (e.g., 2 mM) of thymidine will arrest them at the point in S phase they happen to be. Cells not in S phase when thymidine was added will progress until they reach the next S phase. After a single thymidine treatment, if the cells are released from the arrest for a few hours until they complete S phase and then given a second treatment of thymidine,

they will arrest in late G1 just before the onset of the next S phase (Mitchison 1971b). Hence, a single thymidine block will yield cells arrested throughout S phase, while a double thymidine block will yield cells in late G1 phase. It appears that this was the case for the “G1” (double thymidine block) and “S” phase (single thymidine block) populations analyzed by Stumpf et al. (2013). To obtain their “M” phase cells, Stumpf and colleagues used nocodazole, which interferes with microtubule polymerization and spindle formation, to arrest cells before metaphase (Stumpf et al. 2013). Therefore, this study used only arrested, non-cycling cells for ribosome profiling. In addition to the general issues of unbalanced growth (Mitchison 1971b) and known arrest- specific artifacts (Ly et al. 2015), another major complication is the effects these chemical inhibitors are known to have on the translation machinery (Coldwell et al. 2013).

The second study interrogated translational control in the cell cycle by ribosome profiling using the cyclin-dependent kinase (CDK1) inhibitor RO-3306 to arrest RPE-1 cells, a non-transformed human epithelial line, in the G2 phase (Tanenbaum et al. 2015). Hence, different cells (HeLa vs. RPE-1) and compounds (nocodazole vs. RO-3306) were used in the two human studies (Stumpf et al. 2013; Tanenbaum et al. 2015). To collect their “M” and “G1” samples, Tanenbaum and colleagues washed away the CDK1 inhibitor after an 18-h treatment and collected cells after 45’ (“M”) and 225’ (“G1”). The “G1” samples here were in early G1 because 225’ is not enough time to traverse M and much of the next G1 phase. The overall approach is obviously better than only using arrested cells. Nonetheless, not only it is unlikely that during that relatively short release time the cells resumed “balanced growth”, but also no more than half of all cells progressed in the cell cycle after the inhibitor was removed (Tanenbaum et al. 2015). Furthermore, since CDK1 is known to phosphorylate translation factors [e.g., eIF4G1; (Dobrikov et al. 2014)] it is hard to exclude arrest-induced effects on translation.

Despite the experimental limitations arising from their synchronization strategies, both human studies identified clear examples of translational control in the cell cycle. The common theme in both studies was the coordinate translational regulation of multiple components of the same pathway, such as the mitotic inhibition of PI3 kinase and centriole duplication pathways (Tanenbaum et al. 2015). Stumpf and colleagues pinpointed the upregulation of transcripts encoding proteins of lipid metabolism (M phase), DNA repair (S phase), nuclear transport (G1 and S phase) and chromosome cohesion (M phase) (Stumpf et al. 2013). Both groups went a step further and validated with secondary assays a few cases of translational control, for example, the S phase translational activation of RICTOR (Stumpf et al. 2013) and the disproportional repression of Emi1 in mitosis (Tanenbaum et al. 2015). RICTOR acts positively on mTOR (Sarbasov et al. 2005), arguing that increased levels of RICTOR in S phase may broadly stimulate growth at that stage of the cell cycle. The consequences of repressing translation of *EMI1* in mitosis are directly linked to cell cycle progression. In the G2 phase, Emi1 inhibits the Anaphase-Promoting Complex (APC), so that cells can accumulate mitotic cyclins and enter mitosis (Di Fiore and Pines 2008). To exit mitosis, however, Emi1 must be inactivated, enabling the APC to destroy cyclins. It turns out that previously described ways of controlling Emi1 (e.g., protein stability) are not enough, and translational repression is necessary to bring the levels of Emi1 below the threshold needed to inhibit APC (Tanenbaum et al. 2015). Hence, the disproportionate

repression of Emi1 synthesis in mitosis ensures that little if any Emi1 activity remains at that critical cell cycle transition.

As satisfying as the cases of translational control outlined above may be to evaluate the extent to which the problem of identifying transcripts that are under cell cycle-dependent control in human cells is solved, we need to take a more global view and ask whether the datasets produced in each study are overlapping to a significant extent. But this is where the limitations mentioned above come into play. The two studies queried not only different cell lines with different methods, but also different, non-overlapping phases of the cell cycle (Fig. 1a). Only M phase was examined in both studies, making it the logical starting point for comparative analysis. In mammalian cells, rates of protein synthesis are repressed globally in mitosis, by about 35% (Tanenbaum et al. 2015). Yet both studies showed that mRNAs can be translationally activated even in mitosis. The Tanenbaum study, for example, found 17 mRNAs that were exclusively upregulated in M phase, while many other mRNAs stayed under positive translational control as cells went through mitosis (Tanenbaum et al. 2015). Overall, the Stumpf study found 230 transcripts (Stumpf et al. 2013) and the Tanenbaum study 222 transcripts (Tanenbaum et al. 2015) that were under positive translational control in M phase. Strikingly, only 16 transcripts are common to both datasets (Fig. 1b; DNAJC3, KLHL11, GNL3L, PODXL, ULBP3, CCRN4L, CTGF, THBS1, NDRG3, ATP6V0D1, MKRN3, RNASEK, C20orf29, NUAKE2, SOX9, LDOC1). Likewise, of the 110 transcripts identified by Stumpf and colleagues to be disproportionately repressed in mitosis (of note, EMI1 was not on their list), 32 were also identified in the Tanenbaum study.

It is clear that the overlap between the two studies is dishearteningly low. Why? Although some possible experimental explanations were outlined above, we also need to consider that the datasets were analyzed differently in these studies. The Stumpf study relied on a computational platform, *babel*, they developed (Olshen et al. 2013). The Tanenbaum study defined translational efficiency (TE) as the ratio of ribosome-bound-to total mRNA-bound-reads for a given transcript. Then, they applied fairly stringent cutoffs (>200 mRNA reads; <threefold difference between replicates; >threefold TE differences and >twofold differences in RPKM values of ribosome footprints) to identify differences in TE (Tanenbaum et al. 2015). To determine if differences in the analysis led to the low overlap between the two studies, we used the *babel* platform to analyze the raw read output of the Tanenbaum study. We applied a 200 read cutoff and the *babel* outputs with *p* value <0.01 and false discovery rate (FDR) <0.2. With these cutoffs, using the data of the Tanenbaum study, the *babel* platform identified 95 transcripts that were upregulated in M phase. All but one were also identified as significantly upregulated by Tanenbaum and colleagues (Tanenbaum et al. 2015). We observed similarly high correlations for the G2, and G1 datasets of the Tanenbaum study analyzed with the *babel* platform. Hence, we conclude that the low overlap in the sets of transcripts under translational control in the two studies is more likely to be the result of experimental differences (different cell lines, synchronization, arrest-specific artifacts, etc.) rather than the result of significant differences in the analysis of the raw data. Overall, we argue that the landscape of cell cycle-dependent translational control in the human cell cycle is far from defined. Future studies ought to focus on unperturbed synchronous cultures, covering the entire cell cycle.

Translational control in the budding yeast cell cycle

We recently reported a ribosome profiling study across the entire budding yeast cell cycle (Blank et al. 2017). Instead of using induction synchrony, we relied on a prototypical selection method to obtain synchronous cultures: centrifugal elutriation. Elutriation is a physical method that fractionates an asynchronous cell population by cell size and sedimentation density properties of the cells, with minimal perturbation of cellular functions (Banfalvi 2008; Gordon and Elliott 1977). Elutriation has limitations: it works best for spherical cells; it requires complex instrumentation; being a selection method, the yield of synchronous samples is inherently low. Nonetheless, the synchrony obtained is outstanding. Elutriation works exceptionally well for budding yeast cells, which are relatively spherical and display a wide range of size distributions along the cell cycle. To solve the yield problem, Blank and colleagues generated pools of elutriated samples collected at the same cell size, creating a cell size series spanning the entire cell cycle (Blank et al. 2017). The Blank study pinpointed transcripts with cell cycle-dependent translational efficiency, the first such cases in yeast. However, the dataset was very small: only 17 mRNAs displayed periodic translational efficiency, out of a total of only 55 mRNAs that were translationally regulated. Unexpectedly, translation of mRNAs encoding core enzymes of fatty acid biosynthesis, acetyl-CoA carboxylase (*ACCI*) and fatty acid synthase (*FAS1* and *FAS2*), was upregulated in mitosis (Blank et al. 2017). At least in animal cells, the lipid repertoire is dynamic during the cell cycle (Atilla-Gokcumen et al. 2014). Membrane rearrangements late in the cell cycle (e.g., for cytokinesis and those involving the nuclear membrane) are hallmarks of the eukaryotic cell cycle. In fact, acetyl-CoA carboxylase and fatty acid synthase mutants in yeast fail to appropriately increase the area of their nuclear membrane (Walters et al. 2014). The nuclear membrane displays a massive reorganization during mitosis, and this phenomenon is observed across a wide range of mitotic strategies. These range from the “open” mitosis of human cells (where the mitotic envelope disassembles completely) to the substantial nuclear membrane expansion necessitated by the “closed” mitosis of budding yeast, to the nuclear “rupture” during mitosis of some fungi when the nucleus elongates without nuclear envelope expansion (Ungricht and Kutay 2017). Importantly, manifestations of the last two modes are also evident in human cells when nuclear envelope homeostasis is sacrificed for cell migration and metastasis (Denais et al. 2016; Raab et al. 2016). Hence, the above considerations regarding the physiological significance of this regulation of lipogenic enzymes at the translational level provide reasonable avenues for future studies.

Albeit not validated in detail in the Blank study, transcripts encoding proteins that control chromosome segregation were translationally regulated. The yeast spindle pole body (SPB) is analogous to the mammalian centrosome. The SPB duplicates once every cell cycle, to form the poles of the mitotic spindle (Jaspersen and Winey 2004). At some unknown point in G1, a satellite structure is formed next to the SPB, which nucleates the formation of a new SPB. The two SPBs then separate to opposite sides of the nucleus in mitosis (Jaspersen and Winey 2004). Two pivotal regulators of SPB satellite formation are Dsk2p and Mps1p (Burns et al. 2015; Elserafy et al. 2014). Blank and colleagues discovered that the translational efficiency of *MPS1* and *DSK2* was periodic in the cell cycle (Blank et al.

2017). Translation of *DSK2* was upregulated in mitosis, while the translational efficiency of *MPS1* was the highest in early G1 (Blank et al. 2017). Remarkably, synthesis of Plk4 and CP110, two key regulators of the human centriole (analogous to the yeast SPB), were found to be strongly regulated at the translational level in the Tanenbaum study (Tanenbaum et al. 2015). If validated in future research in both human and yeast systems, these findings raise the possibility that establishment of the mitotic apparatus for chromosome segregation may be a direct output of growth pathways, thereby linking the seemingly continuous cell growth with discontinuous cell cycle landmarks.

Despite these exciting results, the Blank study identified only a very small number of translationally regulated transcripts: 55 in total, of which only 17 were regulated in a cell cycle-dependent manner. Is this an underestimate? As mentioned above, recent statistical analyses showed that in contrast to earlier studies, mRNA levels explain most ($\approx 90\%$) of the variance in protein abundances in yeast and other systems, with translation accounting for $\approx 5\text{--}8\%$ (Albert et al. 2014; Csardi et al. 2015; Li and Biggin 2015). Hence, from the 3291 loci for which the Blank study had transcript and footprint data that passed quality control and entered our analysis, we would have expected to identify $\approx 160\text{--}260$ transcripts under translational control, instead of the 55 transcripts we identified (three- to fivefold reduction). We believe the reason for the lower estimates has to do with biases in the analysis of the data. We used two freely available statistical packages, *babel* (Olshen et al. 2013) and *anota* (Larsson et al. 2011), to analyze our data. The *anota* package uses analysis of partial variance to tackle the problem of spurious correlations arising from increases or decreases in the mRNA populations rather than changes in translation efficiency. Of note, each package identified hundreds (>400) of putative mRNAs under translational control (Blank et al. 2017). To winnow these lists and minimize false positives (Csardi et al. 2015), we focused on mRNA targets identified by both statistical packages. The overlap of these two approaches, against the same raw data, was unexpectedly low: only 55 mRNAs were identified in common (Blank et al. 2017; see Fig. 2). These 55 transcripts are more likely to represent the strongest targets of translational control [indeed, 26 of them have also been reported to be regulated translationally in meiosis (Brar et al. 2012)]. Nonetheless, it likely that we missed many false-negative mRNAs identified by one but not both statistical packages. Hence, at least due to limitations in the analysis, in this case, the picture of translational control in the yeast cell cycle is far from complete.

Conclusions and outlook

The three studies we highlighted above have decisively moved the field forward. Before them, only a few isolated cases of transcripts under periodic translational control were known (Polymenis and Aramayo 2015). Going forward, pressing problems must be solved. Human cells would have to be sampled by selection methods, to overcome the persistent problem of arrest-specific artifacts (Ly et al. 2015). Elutriation has already been used to sample the proteome in the cell cycle of myeloid leukemia cells (Ly et al. 2014). Although elutriation is more challenging than induction methods, there is no reason why elutriation cannot be used for ribosome profiling of human cells. Also, as illustrated by the divergent output of different approaches for the analysis of raw ribosome profiling data (Blank et al. 2017), more work in the analysis platforms would be needed. At a minimum, a robust,

unified platform for the analysis of ribosome profiling data must be adopted by the research community, including the minimum number of replicates and sequencing reads for such studies. None of these obstacles are insurmountable, and it is only a matter of time before the landscape of translational control in the cell cycle comes into full view.

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Fig. 1. Synchronous cell population samples obtained by induction synchrony in human ribosome profiling studies. **a** The *blocks* indicate the presumed cell distributions in the cell cycle (shown with the *black arrows*) used for ribosome profiling of HeLa (Stumpf et al. 2013; *hatched pattern*) or RPE-1 cells (Tanenbaum et al. 2015; *dotted pattern*). It is assumed that the S, or G1, populations in the Stumpf study were collected after a single, or double, thymidine block, respectively. **b** *Venn diagram* showing the poor overlap between the Tanenbaum (*left*) and Stumpf (*right*) studies, regarding the transcripts under positive translational control in mitosis that were identified in each study

Fig. 2.

Statistical evaluation of ribosome footprint and mRNA raw data of elutriated yeast cells.

Venn diagram showing the poor overlap between the *babel* and *anota* platforms, used to identify transcripts under translational control from the yeast cell cycle dataset (Blank et al. 2017)