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From transcriptional complexity to cellular phenotypes: lessons from yeast.

Running title: From transcriptional complexity to cellular phenotypes

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Abstract.

Pervasive transcription has been reported in many eukaryotic organisms, revealing a highly interleaved transcriptome organization that involves thousands of coding and non-coding RNAs. However, to date, the biological impact of transcriptome complexity is still poorly understood. Here I will review how subtle variations of the transcriptome can lead to divergent cellular phenotypes by fine-tuning both its coding potential and regulation. I will discuss strategies that can be used to link molecular variations with divergent biological outcomes. Finally, I will explore the implication of transcriptional complexity for our understanding of gene expression in the context of cell-to-cell phenotypic variability.

Introduction.

One of the biggest challenges in biology is to understand how identical information encoded in the genome generates diversity between cells. Regulation of gene expression is fundamental for cells to adapt to new environmental conditions. However, in addition to thriving in different environments, cells need to differentiate from their peers. This differentiation allows cells to acquire specialized functions or adapt to alternative environments. A clear example is the case of multicellular organisms, where the same genome gives rise to a plethora of specialized cells and tissues. Even inside defined cell types, cell-to-cell differences allow particular cells to diversify their response to external stimuli (*e.g.* explore different phenotypic spaces). Cell-to-cell differences are even more important in unicellular organisms, where each clonal cell is an independent organism. The existence of this heterogeneity allows clonal cells to diversify their phenotype and assure that at least a small proportion of a given population will be prepared to adapt to unforeseen stimuli. This survival strategy is commonly referred as *bet hedging* (Veening et al. 2008; Levy et al. 2012). For example, in microbial communities non-genetic heterogeneity diversifies cellular phenotypes and aids some cells to survive adverse environmental conditions that otherwise would kill the whole population (Kint et al. 2012). Phenotypic heterogeneity allows cells to adapt to variable nutrient or stress conditions (Levy et al. 2012). In mammalians, it has been proposed that phenotypic heterogeneity allows the appearance of drug-tolerant persister cancer cells (*i.e.,* able to escape drug treatment without acquiring genetic mutations)(Sharma et al. 2010; Shaffer et al. 2017). Therefore, this phenomenon could play also a role during the development of complex diseases such as cancer.

Cell-to-cell phenotypic heterogeneity is an advantageous trait that can be selected evolutionary (Fehrmann et al. 2013; Bódi et al. 2017). Multiple factors modulate the appearance of phenotypic heterogeneity, such as gene expression variability,

transcriptional noise or variable epigenetic marks. Transcription is one of the gene expression steps contributing to the appearance of phenotypic heterogeneity. Mechanism underpinning transcriptional noise and increasing variability of gene expression have been extensively reviewed (Liu et al. 2016). In this review, I will focus instead on how even small variations of the transcriptome can contribute to the appearance of phenotypically different subpopulations of cells. The study of such phenomena will help us not only understand better cell-to-cell differences, but also assign functional consequences to subtle variations of the transcriptome. Although this fundamental phenomenon is relevant for all organisms, here I will focus on the study of *Saccharomyces cerevisiae*. Because of its small genome and genetic tractability, *S. cerevisiae* is an ideal organism to study basic mechanisms of eukaryotic gene expression. Its unicellular life style, limited splicing and the fact that it does not possess functional miRNA machinery, facilitates the interpretation of transcriptome variations and its functional validation. First, I will discuss how variations of the transcriptome can contribute to diversify the proteome. Then, I will explore how more subtle variations can also modulate the gene expression process. I will discuss how we can use that information to improve both our understanding of cell-to-cell differences and the transcription process. Finally, I will discuss potential strategies that we can use to link molecular variations with divergent biological outcomes, and discuss the implication of transcriptional complexity for our understanding of gene expression.

Diversifying the proteome.

Variations of the transcriptome can affect cellular phenotypes by diversifying the proteome (Figure 1). A well-known example is alternative splicing, the process by which specific exons can be included in the mature mRNA sequence (Naftelberg et al. 2015). Alternative splicing can lead to the inclusion of protein domains changing protein localization, interaction with ligands or even enzymatic proprieties (Kelemen et al. 2013). Alternative splicing is common in high eukaryotes, however it is relatively rare in *S. cerevisiae* where only a handful of cases are well characterized (Schreiber et al. 2015; Juneau et al. 2009). Alternative splicing diversifies the transcriptome, however up to what degree it contributes to the proteome complexity is not clear (Tress et al. 2017).

Diversification of the coding potential can also be achieved by varying transcript boundaries, namely by the use of alternative transcription start sites (TSSs) or alternative polyadenylations sites (APAs) (Pelechano et al. 2013; Arribere & Gilbert 2013; Malabat et al. 2015; Waern & Snyder 2013). Recently it has been suggested that mammalian transcript isoform differences across tissues are predominantly driven by alternative boundaries of transcription rather than by alternative splicing (Reyes and Huber, https://doi.org/10.1101/127894). One classical example where alternative transcription boundaries change coding potential in *S. cerevisiae* is *SUC2*. *SUC2* encodes a sucrose invertase that depending on the TSSs used will lead to the production of either a secreted or cytosolic protein (Carlson & Botstein 1982). Variations of TSSs leading to differences in the N-terminal regions of proteins have been confirmed by transcriptomic (Pelechano et al. 2013; Arribere & Gilbert 2013) and N-terminal proteomic approaches (Fournier et al. 2012; Helsens et al. 2011; Gawron et al. 2016). Internal translation initiation events (inside annotated coding regions) could be caused by truncated or cryptic transcripts (Carlson & Botstein 1982; Pelechano et al. 2013) or from inefficient translation initiation of upstream start codons. Alternative TSSs can produce N-terminal proteoforms leading to proteins with different stability (Gawron et al. 2016) or even the production of truncated proteins able to act as dominant-negative factors opposing the function of the complete protein (Ungewitter & Scrable 2010). Alternative polyadenylation sites (APAs) can also produce truncated proteins. In some cases APAs produce mRNAs without stop codons that would be targets of nonstop mediated decay

(Roy & Jacobson 2013). However, in other cases APAs can introduce untemplated (Yao et al. 2012) or cryptic (Ni & Kuperwasser 2016) stop codons. For example, in human cells, an untemplated stop codon (*i.e.* not present in the DNA sequence) arises from APA and generates a C_t truncated tRNA synthetase (Yao et al. 2012). Similar phenomena have been proposed to occur in budding yeast cells (Pelechano et al. 2013; Georis et al. 2015). However, the widespread usage of APAs still makes difficult to distinguish an event introducing novel stop codons from the noise associated to the polyadenylation process itself. Independently of this, the previous examples show that transcriptome variability contributes to proteome diversity. This effect is even increased when considering that transcript boundaries vary significantly across environmental conditions even inside apparent homogeneous populations of cells (Pelechano et al. 2013; Waern & Snyder 2013; Yoon & Brem 2010; Wilkening et al. 2013). Another process that can also diversify the proteome is the use of directed frame shifts during the translation process (Gerashchenko et al. 2012; Meydan et al. 2017). Although there are some clear examples, their global contribution is difficult to assess. One of the reasons is that most of our transcriptomic approaches are based on the bulk analysis of mRNA populations. Therefore, frame-shifts affecting a small fraction of the mRNA molecules (or even in particular cells) will be hard to find in the background of population-wide mRNA measurements.

In addition to variations of canonical open reading frames (ORFs), other less well-characterized transcripts can be translated. Of special interest is the case of short ORFs (sORFs) producing peptides (Andrews & Rothnagel 2014). sORFs can originate from upstream ORFs (uORF, translation regulatory regions located in the 5'UTR of mRNAs), from putative non-coding RNA or even from more complex arrangements of overlapping transcription units (Andrews & Rothnagel 2014; Pelechano et al. 2013; Mackowiak et al. 2015; Carvunis et al. 2012). Pioneering analysis of the biological function of sORFs was performed using budding yeast (Kastenmayer et al. 2006). Those approaches were limited by the available genetic tools at that time and by the proximity of sORFs to other coding and regulatory regions of the genome. The use of novel approaches in the coming years, such as single nucleotide DNA modifications or directly targeting RNA or peptide molecules without affecting the gene loci, will likely allow a finer dissection of sORF function and regulation.

Modulating gene expression.

Beyond shaping the expressed proteome, transcription complexity has an effect on gene expression regulation (Figure 1). Transcription, or the produced coding and non-coding transcripts, can regulate gene expression by processes such as transcriptional interference, chromatin remodeling, spreading of regulatory signals, protein scaffolding or the production of antisense transcripts. These processes have been extensively reviewed before (Pelechano & Steinmetz 2013; Jensen et al. 2013; Engreitz et al. 2016). Here I will focus on the transcripts variations that can change the proprieties of these transcripts without affecting their coding potential. We can consider that the mRNAs produced from a given gene are often not identical, but represent multiple variants (*e.g.* isoforms) that can respond differentially to changing environments.

RNA molecules can differ in their stability and sensitivity to nuclear or cytoplasmic decay (Jensen et al. 2013). Alternative TSSs can lead to the appearance of mRNA isoforms containing upstream ORFs (uORF) in the 5'UTR that targets mRNAs to degradation by Non-sense mediated decay (NMD) (Malabat et al. 2015). Similar processes can occur also in non-coding RNAs, where the extension of their 3' boundaries can diversify the used RNA decay pathway (Wery et al. 2016; Marquardt et al. 2011). Differences in used APAs also diversifies mRNAs changing their stabilities and ability to

interact with RNA binding proteins (Gupta et al. 2014; Geisberg et al. 2014). Differences in RNA stability have profound implications for gene expression, and affects both RNA abundance and its dynamics. For example, after changes in transcription rate, mRNAs with shorter half-lives will reach faster new equilibriums and thus facilitate the adaptation to changing environments (Pérez-Ortín et al. 2007). Therefore two cells expressing alternative isoforms with different stabilities for the same gene, would be able to adapt at different velocity to a new environment.

Variations in the transcriptome can also affect how mRNA molecules are recognized by ribosomes. Secondary 5'UTR structures or uORFs are well-known phenomena regulating mRNA translation that depend on the choice of TSSs (Malabat et al. 2015; Arribere & Gilbert 2013; Hinnebusch et al. 2016). Interestingly differential translation regulation according to the used TSSs can lead to divergent outcomes depending on the environmental conditions (Tamarkin-Ben-Harush et al. 2017). In addition to well understood variation of 5'UTRs, other more complex structures such as polycistronic transcripts have also been found to be present in multiple eukaryotic genomes (Gordon et al. 2015; Pelechano et al. 2013). More research would be needed to understand if polycistronic mRNAs are translated to produce functional proteins or targeted to cytoplasmic RNA decay. In other cases, variation of the transcriptome can control the subcellular localization of the encoded proteins (Berkovits & Mayr 2015). All these examples show the current limitations of extrapolating protein abundance from mRNA abundance, especially in the context of environmental transitions.

In addition to differences in sequence, RNA molecules can also differ in other aspects such as nucleotide modifications and structure. Interaction with particular RNA binding proteins can generate subcellular RNA aggregates modifying RNA post-transcriptional life (Protter & Parker 2016). Thanks to the development of new methods allowing the genome-wide study of RNA modifications, there has been a rise in the study of the epitranscriptome (Helm & Motorin 2017). For example *N*6-methyladenosine (m^6A), one of the most abundant internal modifications of eukaryotic mRNAs, has been linked with changes in mRNA structure, maturation and translation (reviewed in (Zhao et al. 2017; Meyer & Jaffrey 2014)). Variations in RNA structure and nucleotide modifications can also modulate their interaction with the cellular machinery (Lewis et al. 2017). Therefore differences in RNA structure and the epitrascriptome can further diversify transcriptome functional potential. In that context, it is possible to hypothesize that some of these factors (or their interaction) could also contribute to the observed coupling between nuclear and cytoplasmic processes where the transcription process can influence mRNA post-transcriptional life in the cytoplasm (Choder 2011; Lewis et al. 2017).

Assigning phenotypes to molecular variations.

Although subtle variations of the transcriptome can have biological effects, measuring the extent to which those variation modulate cellular phenotype remains challenging. There are multiple approaches to link phenotypes with molecular variations such as: measuring the cellular behaviour of individual molecules, their association to particular cellular phenotypes or evolutionary conservation. All these approaches rely on the assumption that biologically relevant variations at cellular level are also likely to present divergent molecular behaviours and to be evolutionary conserved.

The development of isoforms-specific and epitranscriptomic approaches has allowed us to assign cellular behaviours to different molecules (*i.e.* measuring differential interaction with the cellular machinery). In general, these methods are based on the combination of novel genome-wide approaches with classical molecular biology techniques. For example, isoform-specific mRNA stability have been measured

combining transcriptional inhibition with APAs identification (Gupta et al. 2014; Geisberg et al. 2014). Potentially, isoform-specific measures can also be combined with metabolic labelling to obtain measures of transcription rate and mRNA decay independently of transcriptional inhibition (Sun et al. 2012). Differences in translation ability depending on the used TSSs (Arribere & Gilbert 2013) or isoform-specific interactions with RNA binding proteins (Gupta et al. 2014) have been measured using polyribosome purifications or RNA binding proteins immunoprecipitation respectively. Using cellular fractionation is now possible to study subcellular localization of mRNA isoforms (*e.g.,* mitochondria- or chromatin-associated mRNAs (Marc et al. 2002; Carrillo Oesterreich et al. 2010)) or the variation of any of the mentioned parameters during environmental transitions.

In addition to directly measure the properties of the transcripts, we can characterize them according to their interaction with the cellular machinery. Transcripts have been classified according to their response to the disruption of elements of the degradation or regulatory pathways (Malabat et al. 2015; Van Dijk et al. 2011; Wery et al. 2016; Xu et al. 2009; Jensen et al. 2013). These studies inform us on the sensitivity of each molecule and point to variations of the transcriptome more likely associated to different cellular responses.

We can also assign phenotypes to molecular variations by studying their association to particular cellular states. Transcripts have been classified according to their abundance in different environmental conditions or stress responses (Pelechano et al. 2013; Waern & Snyder 2013; Yoon & Brem 2010; Wilkening et al. 2013). Potentially we can also sort individual cells according to their particular features (*e.g.* using flow cytometry) and study the transcriptome of more homogeneous populations. It is not difficult to imagine that single-cell isoform-specific measures of transcription, that have been successfully applied to mammals (Islam et al. 2013; Velten et al. 2015), will be applied in the future to budding yeast. However up to this date single-cell RNASeq approaches in *S. cerevisiae* remain challenging due to its small cellular size and cell wall. The combination of all these approaches will allow us to characterize variations of the transcriptome according to their coordinated regulation. The underlying hypothesis for all these approaches is that transcripts expressed in a coordinated way across environments or perturbation are more likely to be involved in the same process. Studies that assign function based on *guilt by association* have been successfully applied to the study of ncRNAs (Guttman et al. 2011). To successfully extend those approaches to isoform- and epitranscriptome-specific variations it will be necessary to integrate the wealth of available transcriptomic datasets in budding yeast and their expression across conditions.

To complement these approaches, and prioritize variations of the transcriptome more likely to be functionally relevant, we can use evolutionary criteria. Budding yeast is an ideal organism to study evolutionary conservation of the transcriptome due to its small genome, and the availability of genetic tools and well characterized related species (Scannell et al. 2011). The study of close related budding yeast species allows to explore the evolution of molecular mechanism underlying their phenotypic diversity (Skelly et al. 2013). Another example of how evolutionary criteria can improve our understanding of RNA-related mechanism, is the discovery that RNA interference mechanism loss in particular yeast species could be explained by the acquisition of killer virus conferring advantageous adaptations (Drinnenberg et al. 2011). In addition, it is possible to use yeast artificial chromosomes (YACs) to compare the behavior of *different genomes* in the same cellular context (Moqtaderi et al. 2013). Measuring the cellular behavior of individual mRNA molecules in related species will allow assessing if the transcriptome variations and their associated functional differences are conserved. In the same way studying the different response of related species to the same environmental challenges will also inform us about the conservation of the divergent regulation of particular

variations of the transcriptome. Another possibility to assign biological function to alternative isoforms is to study the differences between members of the same species and to perform quantitative trail loci (QTL) analysis (Cannavò et al. 2016; Schor et al. 2017).

To complement these approaches, it will be necessary to develop novel high-throughput methods to specifically regulate the expression of isoforms or epitranscriptomic-specific molecules without affecting the neighboring transcripts. Those approaches will be necessary to selectively target specific molecules (*e.g.* isoforms) and thus measure their impact on cellular phenotype both at single-cell and population level. Recent developments, such as those associated to CRISPR technologies, would likely allow the required molecular precision in the coming years (Shalem et al. 2015). In particular, the introduction of single nucleotide mutations and the use of dCas9-based strategies to modulate transcription activity (*i.e.* without modifying the genome) can be expected to facilitate the study of the transcriptome complexity in the near future.

Conclusions.

Subtle variations of the transcriptome can have a biological impact. However, to-date it is not clear whether most differences contribute to diversify cellular phenotypes, or whether only a fraction of those variations have functional impact. More research will be needed to determine which variations of the transcriptome are functionally equivalent (*e.g.* just product of the intrinsic noise of the gene expression process); and which variations contribute to the appearance of divergent phenotypes. By studying the functional consequences of small variations of the transcriptome, we will improve our understanding of transcription regulation and the complex life of the mRNA. Budding yeast is the ideal eukaryotic organism to pilot this study, due to its extremely well characterized genome and availability of genetic and genomic tools. The study of this general process will likely have a general impact in our understanding of biology. The recent development of single-cell transcriptomic approaches has allowed us to improve our understanding of gene expression. In a similar way, I expect that a better understanding of the impact of subtle variations on the transcriptome will allow us to understand better how cells fine-tune their transcriptional responses. And potentially how evolution has harnessed these variations to contribute to the appearance of cell populations with defined transcriptomic programs and divergent cellular phenotypes.

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Figures

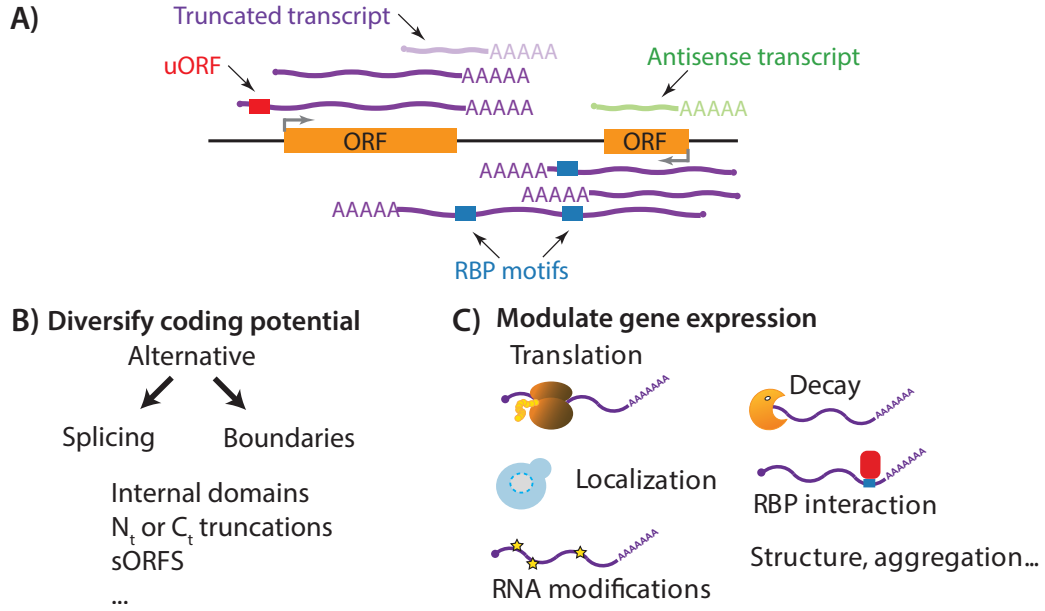

Figure 1. Sources and impact of transcriptome diversity. Examples of alternative mRNA molecules generated (A) and their impact on protein coding potential (B) and diversification of gene expression regulation (C).

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Conflict of Interest.
The author declares that there is no conflict of interest.