# Mitochondria and the non-genetic origins of cell-to-cell variability: More is different

*Raúl Guantes[1)]*, Juan Díaz-Colunga[2)] and Francisco J. Iborra[2)]**

**Gene expression activity is heterogeneous in a population of isogenic cells. Identifying the molecular basis of this variability will improve our understanding of phenomena like tumor resistance to drugs, virus infection, or cell fate choice. The complexity of the molecular steps and machines involved in transcription and translation could introduce sources of randomness at many levels, but a common constraint to most of these processes is its energy dependence. In eukaryotic cells, most of this energy is provided by mitochondria. A clonal population of cells may show a large variability in the number and functionality of mitochondria. Here, we discuss how differences in the mitochondrial content of each cell contribute to heterogeneity in gene products. Changes in the amount of mitochondria can also entail drastic alterations of a cell's gene expression program, which ultimately leads to phenotypic diversity.**

**Keywords:**
alternative splicing; gene expression noise; non-genetic variability; transcription

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[1)] Department of Condensed Matter Physics, Materials Science Institute 'Nicolás Cabrera' and Institute of Condensed Matter Physics (IFIMAC), Universidad Autónoma de Madrid, Campus de Cantoblanco, Madrid, Spain
[2)] Centro Nacional de Biotecnología, CSIC, Campus de Cantoblanco, Madrid, Spain

***Corresponding authors:**
Raúl Guantes
E-mail: raul.guantes@uam.es
Francisco J. Iborra
E-mail: fjiborra@cnb.csic.es

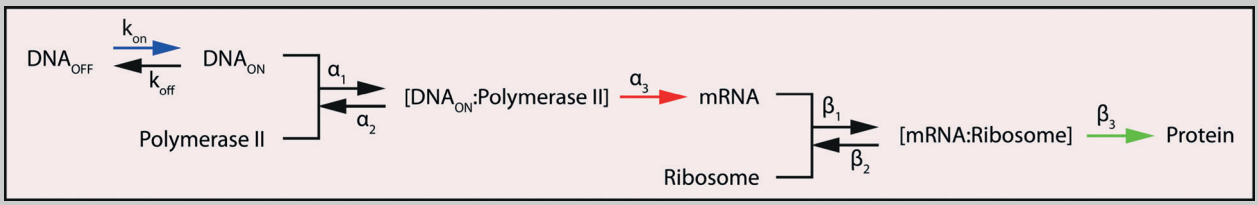
**Abbreviations:**
**AS,** alternative splicing; **mtDNA,** mitochondrial DNA.

## Introduction

Cells of a clonal population in a homogeneous medium may show large variations in size, morphology, molecular components, and activity. This cellular heterogeneity plays important functional roles in processes such as development and cell differentiation, cell decisions, virus infection, apoptosis, and cancer [1–5]. Cell-to-cell variability is originated by differences in gene activity, which confers each cell a unique "expression fingerprint" with possible phenotypic consequences. But, can we trace back the primary causes of these differences in gene activity?

Gene expression entails the controlled and ordered assembly of basic molecular components (nucleotides and aminoacids) into nucleic acids and proteins. This complex process involves the concerted action of many different molecular steps (Box 1) regulated by a plethora of enzymes and protein complexes: chromatin remodeling factors, transcription factors, polymerases, ribosomes, etc. One can then envision two fundamental sources of variability in gene expression: variability in the number of molecular constituents, both metabolites and components of the gene expression machinery, and variability in the biochemical reactions involved in the gene expression cycle [6]. Indeed, both sources are not independent [7], even though they are often treated as separate contributions [8, 9]. Biochemical reactions can be considered as probabilistic events creating spontaneous fluctuations in mRNA and protein abundances, while cell-to-cell differences in molecular components are assumed to change the rates at which these reactions take place [10].
Energy, mostly in the form of ATP, is needed both to synthesize the metabolites and the molecular machinery that fuels gene expression, and to overcome free-energy barriers in many of the biochemical reactions involved [11]. In fact, gene expression is an energy demanding process, with 75% of the cellular ATP budget invested into mRNA and protein polymerization [12, 13]. Many eukaryotes, prominently normal differentiated cells in metazoan organisms, produce most of their ATP through oxidative phosphorylation in mitochondria [14]. By enabling oxidative phosphorylation across a wide

## Box 1




The central dogma of molecular biology can be phrased as a series of biochemical reactions involving gene activation/inactivation ($DNA_{on}/DNA_{off}$), transcription initiation (with binding/unbinding of the RNA Pol II) and elongation to synthesize a mRNA strand, and translation initiation (binding/unbinding of mRNA to the ribosome) and elongation in ribosomes to synthesize protein. Some of these processes are highly dependent on ATP (notably gene activation, in blue, transcription elongation, in red, and translation elongation, in green, see Fig. 1).

This series of reactions can be translated, using mass action kinetics, in a system of differential equations for the evolution of mRNA and protein. Using the equilibrium approximation for the complexes [$DNA_{on}$:Polymerase II] and [mRNA:Ribosome] and defining effective transcription ($k_m^{eff}$) and translation ($k_p^{eff}$) rate constants, the model can be cast in a simplified form, analogous to the "Central Dogma" basic model in Fig. 2:

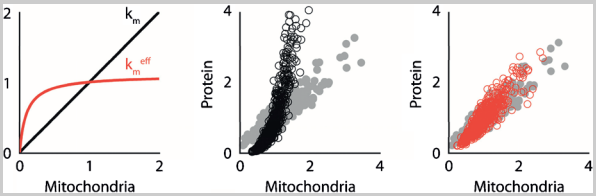
$$\frac{d}{dt}\mathrm{mRNA} = \underbrace{\left[\alpha_1 \cdot \frac{\frac{\alpha_3(\mathrm{ATP})}{\alpha_2}}{\frac{1+\alpha_3(\mathrm{ATP})}{\alpha_2}} \cdot \langle \mathrm{Pol\,II} \rangle\right]}_{k_m^{eff}} \cdot \mathrm{DNA_{ON}} - \gamma_m \cdot \mathrm{mRNA}$$

$$= k_m^{eff}(\mathrm{ATP}) \cdot \mathrm{DNA_{ON}} - \gamma_m \cdot \mathrm{mRNA} \qquad (1)$$

$$\frac{d}{dt}\mathrm{Protein} = \underbrace{\left[\beta_1 \cdot \frac{\frac{\beta_3(\mathrm{ATP})}{\beta_2}}{\frac{1+\beta_3(\mathrm{ATP})}{\beta_2}} \cdot \langle \mathrm{Ribosome} \rangle\right]}_{k_p^{eff}} \cdot \mathrm{mRNA}$$

$$- \gamma_p \cdot \mathrm{Protein} = k_p^{eff}(\mathrm{ATP}) \cdot \mathrm{mRNA} - \gamma_p \cdot \mathrm{Protein} \qquad (2)$$

Note that if in the basic Central Dogma model the transcription rate, $k_m$, is identified with the elongation constant, its dependence with mitochondria (and ATP) is linear, but in the model above including binding/unbinding of Pol II and initiation, the dependence of $k_m^{eff}$ with mitochondria is non-linear (left panel below, red line). Stochastic simulation of protein abundance with the model in Fig. 2, using a linear dependence with mitochondria for $k_{on}$, $k_m$, and $k_p$ (Fig. 1C–E) shows a non-linear correlation of protein abundance with mitochondria (middle panel, black circles). Gray circles are experimental measurements. Using the effective rate constants $k_m^{eff}$ and $k_p^{eff}$ defined above, the covariation of protein levels in single cells and mitochondria is linear, in agreement with experimental observations (right panel, red circles).




area of inner membranes, mitochondria raise the energy power of the cell several orders of magnitude, that has led to the hypothesis that mitochondrial genes are responsible for the leap in genome complexity observed between prokaryotes and eukaryotes [13]. The "average" energy budget is highly variable from cell to cell in an isoclonal eukaryotic population, spanning typical ranges between 1 and 10 mM on average. Moreover, the intracellular ATP levels are not equally distributed among the different cellular compartments [15]. Variability in energy budget may thus be a primary cause for cell-to-cell differences in gene expression.

In this essay, we elaborate along these lines discussing the implications of heterogeneity in mitochondrial content, a natural source of energy variation in eukaryotes, on cell-to-cell variability. We first review the central ideas that have been consolidating in the field of non-genetic variability, distinguishing sources of cell-to-cell differences, and emphasizing operational definitions from experimental observables. We then place the role of energy and mitochondria in context, connecting with the current knowledge of how different steps of the gene expression cycle generate variability in mRNA and protein abundance. We show how mitochondria can be viewed both as a "control knob" that amplifies in a graded manner the output of some gene expression processes, and as a non-linear device yielding unpredictable outcomes in other processes, notably in alternative splicing. We finish with a discussion on the implications of mitochondrial heterogeneity in physiology and disease.

## The nuts and bolts of cell-to-cell variability

Phenotypic variability has been identified at the single cell level in many instances, such as cell fate choice and cell differentiation [16], virus infection [3, 17], or drug resistance [4] to name a few. To link phenotypic and molecular variability,

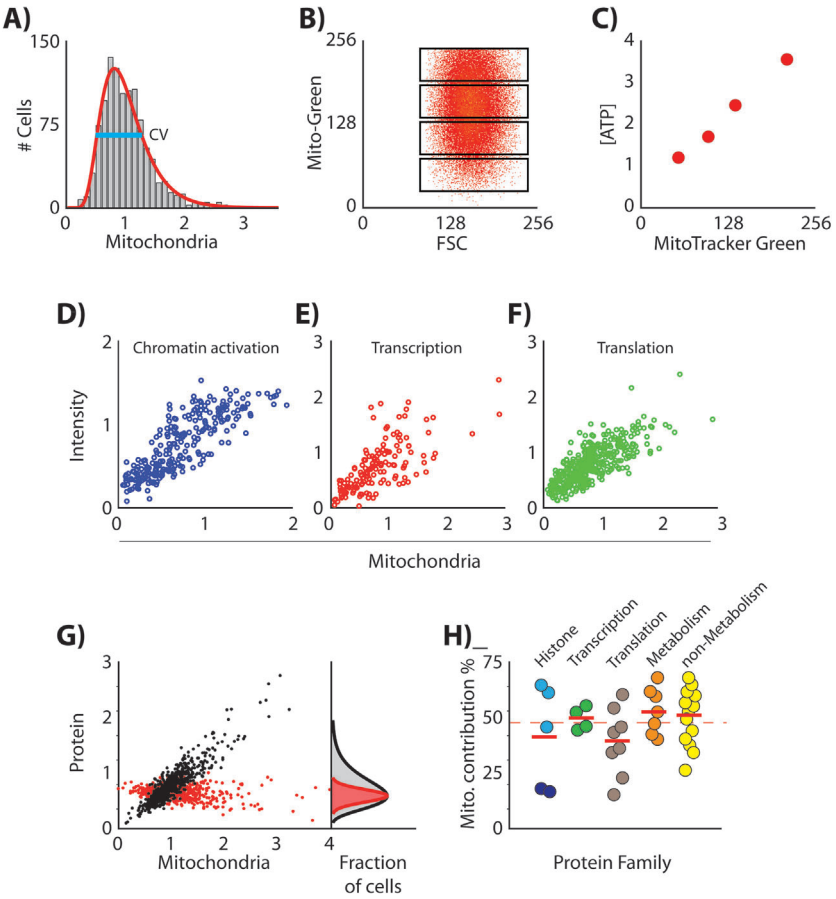



**Figure 1.** Mitochondrial cell-to-cell variability and its contribution to gene expression. **A:** Distribution of mitochondrial levels (normalized by the average value) as quantified by the reporter MitotrackerGreen in a clonal population of HeLa cells. Red line is a fit to a log-normal distribution (most protein distributions also follow this shape). The blue line is twice the standard deviation, from which the coefficient of variation (CV) is obtained (CV = 0.39). **B:** Cells sorted according to mitochondrial content in four different subpopulations (boxes). **C:** ATP content was determined in these fractions and corrected by the number of cells to give an approximate ATP concentration per cell. This is consistent with the high OXPHOS activity previously observed in these cells, which in our culture conditions accounts for ~80% of ATP production [48, 121] **D:** Correlation of mitochondrial levels with amounts of the histone methylation mark H3K4me3, related to promoter activation. Each circle is a cell. **E:** Correlation of mitochondrial levels with transcriptional activity as reported by the amount of nascent RNA, Br-RNA. **F:** Correlation of mitochondrial levels with translation activity (nascent protein) as measured by the precursor AHA. **G:** Mitochondrial levels co-vary with the amount of total protein in cells. The contribution of mitochondria to total cell-to-cell variability (gray distribution) can be obtained by removing the covariation between protein and mitochondria (red distribution, representing variability due to intrinsic and extrinsic sources different from mitochondria). **H:** Quantification of mitochondrial contribution to variability in: histone acetylation and methylation marks related to gene activation (pale blue circles) and gene inactivation (dark blue circles); processes related to transcriptional activity (green circles); processes related to translation activity (brown circles); levels of proteins involved in energy metabolism (orange circles) and levels of proteins non-related to energy metabolism (yellow circles). Dashed line is the average contribution to all protein families, while short red lines indicate average value of each family.

one should simultaneously quantify both and establish causal relationships between them [18]. This has been done in many cases, where variability at the phenotypic level has been connected to some sort of "randomness" or stochasticity in key proteins or genetic circuits.

Cell-to-cell variability in gene expression products can be quantified from the intensity distribution of a fluorescent reporter in a population of cells, as measured by single-cell microscopy or flow cytometry (Fig. 1A and B). The experimental observable in each cell is the "average" level of the readout of interest. Simple statistical measures of dispersion, such as the coefficient of variation (CV, standard deviation relative to mean) or the Fano factor (variance relative to mean), Fig. 1B, are appropriate measures of variability [19]. Using time-lapse microscopy single cells can be tracked in time, providing useful dynamic information of the gene expression process [20].

Early work at this stage used a clever construct to tease apart different sources of randomness operating in cells: two fluorescent reporter genes of equivalent intensity distributions controlled by identical promoters [21]. This experimental setup allows to decompose the total variability into a sum of an "intrinsic" component representing the fluctuations in the expression levels of genes under identical regulation in the same cell, and thus exposed to the same internal environment, and an "extrinsic" component representing the cell-to-cell variation in the expression of identical genes. Intrinsic variability or "intrinsic noise" was identified with the inherent randomness of the biochemical processes leading to gene expression, setting a fundamental physical limit to the precision of gene activity, and in this case it is properly called "noise." On the other hand, extrinsic variability or "extrinsic noise" was attributed to fluctuations in the levels of other cellular components, external to the gene, that have an impact on the gene activity: transcription and signaling factors, cell cycle state, etc. This last contribution represents in fact the internal state of each cell and as such the term "noise" is questionable here. Differences in internal state may well be due to different states of regulation of internal components [22, 23]. Several genome-wide studies adopted the dual reporter strategy to dissect these two different sources of noise in yeast [24] and *E. coli* [25], and found that for relatively abundant proteins, extrinsic factors contributed more than intrinsic ones.

Recent advances in single-cell and single-molecule experimental techniques are expanding our understanding of the molecular mechanisms underlying cell-to-cell variability [26]. The prevailing view is that gene expression is noisy due to bursts of transcriptional activity [19], and this

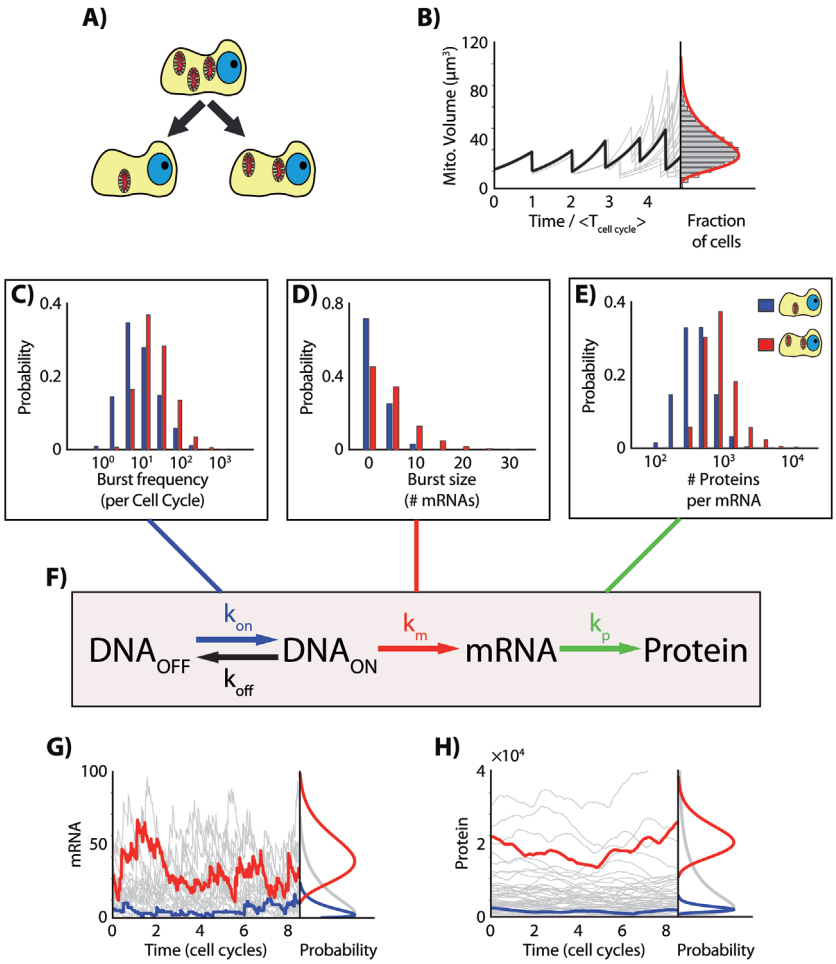



**Figure 2.** Asymmetric segregation of mitochondria and its effect on gene expression variability. **A:** At cell division, different amounts of mitochondria are inherited by daughter cells. **B:** A model with cell-cycle fluctuations and binomial segregation of mitochondria between daughter cells [46] reproduces the log-normal distribution of mitochondria in the final population (right panel, CV = 0.41, compare to Fig. 1B). **C:** Distributions of transcription bursts frequency with different promoter activation rates ($k_{on}$), according to the amount of histone marks in cells with low (blue) and high (red) amounts of mitochondria, as measured in Fig. 1D. **D:** Distributions of mRNA burst sizes with different transcription rates $k_m$, according to the amount of transcriptional activity measured in cells with low and high mitochondrial content (Fig. 1E). **E:** Distributions of protein burst sizes with different translation rates $k_p$, as reported by the differences in translation activity between cells with low and high mitochondrial levels (Fig. 1F). **F:** Basic "Central Dogma" model used for stochastic simulations. The model parameters are taken from average values measured in HeLa cells, and modulated according to the correlations between mitochondria and gene activation, transcription, and translation observed experimentally (Fig. 1D–F). **G:** Stochastic simulations of mRNA amounts in a population of cells with heterogeneous amounts of mitochondria (according to Fig. 1A). Each gray time series represents a cell. The values for $k_{on}$, $k_m$, and $k_p$ have been chosen according to the cell's mitochondrial content. The red line represents a cell with a large amount of mitochondria, and the blue line a cell with low mitochondrial levels. The corresponding total distribution (gray), distribution with high (red) and low (blue) mitochondria are shown on the right. **H:** Simulated protein copy numbers in a population of cells with heterogeneous amounts of mitochondria. Parameters are the same as those used in panel G.

mechanism is common to bacteria, yeast, and animal cells [27–30]. Although promoter architecture may determine to some extent gene specific bursting [31, 32], bursting kinetics seems to be largely influenced by general biophysical constraints [27, 30, 33]. These constraints would operate at a genome-wide level inside each cell and could modulate individual transcription kinetics [34].

## Gene expression and energy constraints

Different factors that are variable from cell to cell could be imposing global constraints on gene expression: the availability of basic metabolites and enzymes needed for protein synthesis [35], the amounts of cellular components of the transcription and translation machinery, notably ribosomes [36], the cell mass and growth state [37], or changes in metabolic reactions [38]. Many of these factors are in fact related: nutrient availability is coupled to metabolism, which is linked to gene expression and growth rate through reallocation of different cellular resources [39, 40]. A common link connecting these factors is the energy available per cell, which is needed for the gene expression process itself, but also impacts on the cell cycle, ribosome synthesis, and mediates changes in metabolic reactions [41].

We have previously shown that a clonal population of HeLa cells displays a natural variability in the amount of mitochondria (Fig. 1A), and that this produces cell-to-cell differences in ATP content [42] (Fig. 1B and C). Heterogeneity in mitochondrial mass has also been observed in hematopoietic stem cells [43] and in solid tumors [44]. There is evidence that cell-to-cell heterogeneity in mitochondrial content stems from asymmetric apportioning of individual mitochondria at cell division [42, 45, 46]. This asymmetric segregation process is similar to the random split of a given number of elements in two subsets, called "binomial partitioning:" mitochondria can be thought of as doing a coin flip to decide which daughter they will go to. This simple statistical model is consistent with experimental data of how proteins are distributed among cells after many cell divisions [47], distributions of cellular mitochondrial content [46] (see also Figs. 1A and 2B) and partition of mitochondrial DNA (mtDNA) at cell division [45].

To elucidate the role of mitochondrial variability on gene expression, one can quantify simultaneously in single cells the mitochondrial levels, and the amounts of proteins, mRNAs, or different markers of transcription and translation activities [48]. Gene expression activity reporters show a strong correlation with mitochondrial content (Fig. 1D–F), which

results in a covariation of mitochondrial levels with protein abundance in individual cells (Fig. 1G). This causes a large coefficient of variation in the protein distribution of the population (Fig. 1G, gray distribution). The contribution of the mitochondria to the total protein variability can be obtained by removing the covariation along the diagonal (Fig. 1G, red circles and red distribution), a procedure similar to calculating the fraction of protein variance explained by mitochondrial levels.

In the following, we will discuss the possible role played by energy and mitochondrial content on several steps of the gene expression cycle, and on the variability of other global factors that change gene expression. The effect of mitochondrial content on mRNA and protein fluctuations will be illustrated with numerical simulations of a "Central Dogma" model of stochastic gene expression (Fig. 2F), with parameter values estimated from experimental measurements in HeLa cells.

## Chromatin and nucleosome organization

Nucleosomes are the basic repeating units of eukaryotic chromatin, and play a critical role in the control of gene expression by competing for DNA binding with many transcription factors. Both nucleosome positioning and competition with transcription factors are dynamic processes [49]; interestingly, while many DNA binding proteins have binding/unbinding times of the order of seconds, the dwell times of histones are in the range from minutes to hours [42, 50]. Transcriptional bursting is the result of long episodes of active gene transcription (gene ON, Fig. 2F) that produce many mRNAs, followed by periods of gene silencing. It is then natural to associate chromatin remodeling and nucleosome repositioning with transcription bursts [51], and several recent studies have indeed revealed a stochastic process of nucleosome assembly and disassembly that may be responsible for transcriptional bursts [52] and cell-to-cell variability in gene expression [53].

Transcriptional bursts can be characterized by two parameters: burst frequency (number of activation events per unit time, Fig. 2C) and burst size (number of transcripts produced per gene activation event, Fig. 2D). Both burst size and frequency contribute to gene expression noise, but differently [30]. Studies with mutated promoters suggest that competition between nucleosomes and transcription factors for binding underlies burst size and frequency modulation: burst size is a characteristic feature of each promoter, and strongly depends on the interaction between the TATA box and proximal nucleosomes [31]. On the other hand, burst frequency is mainly affected by nucleosome positioning alone [54]. Consistent with this, a recent study in which a reporter gene was integrated at random locations in the genome, found that nucleosome occupancy close to the transcription start site controls burst frequency and gene expression variability [55], whereas burst size independently modulates mean expression.

How can mitochondria affect transcriptional bursting? Chromatin remodeling, the local rearrangement of nucleosomes structures, is in many cases mediated by molecular machines that use the energy produced by ATP hydrolysis to change nucleosome positions [56, 57]. Moreover, mitochondria control the levels of Acetyl coA and SAM, essential cofactors for histone modifications, as well as NAD+, which is used by SIRTUINs to catalyze histone deacetylation [58]. Mitochondrial content could also be affecting the amount of histone acetyltransferases, the enzymes responsible for histone acetylation and deacetylation.

We have found that mitochondrial levels largely co-vary with chromatin modification marks related to transcriptional activation [48] (Fig. 1D and H). This correlation can provide a mechanism to effectively modulate the gene activation rate, $k_{on}$, changing the burst frequency distribution, as illustrated with numerical simulations in Fig. 2C.

## Transcription initiation and elongation

The next step in the gene expression cycle after chromatin remodeling is transcription initiation and elongation. First, the transcriptional machinery requires the assembly of large protein complexes at a gene's promoter. Transcription factors and components of the transcription machinery at the promoter have fast turnover rates (from seconds to minutes), enabling multiple rounds of initiation without memory between different initiation events [59]. This suggests a model with inefficient and stochastic transcription initiation, which in vivo measures of RNA polymerase dynamics seem to confirm [60]. On the other hand, elongation, which is energy dependent, is more likely a deterministic process [59]. We found that transcription elongation, as monitored by the amount of the immediate transcription precursor BrU, is highly dependent on mitochondrial content [42] (Fig. 1E). Therefore, the transcription elongation rate, $k_m$ in Fig. 2F, can modulate burst size in a mitochondrial-dependent manner, as numerical simulations show (Fig. 2D).

The cellular levels of BrU are a proxy for the average transcriptional activity in a cell, but its dependence on mitochondrial content can be due to different effects: with more mitochondria, there could be more RNA Pol II molecules engaged in elongation, a higher number of genes being actively transcribed, or a combination of both. The transcription cycle by RNA Pol II can be decomposed into steps with different kinetics, Box 1: binding of RNA Pol II to the DNA (initiation), making a complex that can be abortive, or that can proceed into elongation mode after being modified. Once RNA Pol II elongating molecules complete the transcription cycle, they become free and diffuse throughout the nucleoplasm to start a new transcription round. The rate constants for these processes can be deduced from fluorescence loss in photobleaching (FLIP) experiments, using RNA pol II fluorescent reporters [60]. An analysis of the different kinetic processes in control cells and ATP-depleted cells, shows a much stronger ATP dependence of the elongation rate constant than the initiation constant [48], suggesting a model where elevated mitochondrial content increases both the number of genes actively transcribed and the number of RNA Pol II molecules engaged in elongation.

Further experimental work will be needed to elucidate the molecular mechanisms that link mitochondrial content to transcription initiation and elongation, and the contribution

of RNAP changes to gene expression noise [61]. Imaging superresolution techniques are a promising tool to probe RNA Pol II dynamics and localization with single molecule sensitivity [62], and this technique could be used in concert with reporters of ATP or mitochondrial content.

## Cell cycle and growth

Since gene expression may be coupled to cell growth and division, a possible source of variability in a cell population comes from cell-to-cell differences both in cell-cycle periods and state. The cell cycle can contribute in several ways to cell-to-cell heterogeneity in protein and mRNA: one possible cause of variation is the dependence of protein production on cell-cycle phase [63–65], mainly by increasing transcription in the S/G2/M phase. Interestingly, gene expression and growth can be highly coordinated, so that protein and mRNA *concentrations* (number of copies per cell volume) are invariant along the cell cycle [65, 66]. On the other hand, cell population dynamics generates asymmetric partition of molecular components (to be discussed below) that generates a "noise floor" for extrinsic variability in a cell population [67]. A third possible role of the cell cycle on protein variability is in setting the time scale at which fluctuations decay [47]. Intrinsic fluctuations are typically faster than cell-cycle periods, while fluctuations in global cellular components decay on the time scale of one cell cycle.

In mammalian cells proliferating in normal tissues, cell size and cell-cycle are tightly coupled, and there must be control mechanisms to limit cell size variability [68]. Interestingly, a recent study has uncovered that mitochondria may regulate the balance between cell size and proliferation in mouse liver cells [69]. Cell growth and division depend on biosynthetic capabilities [36]. Mitochondrial levels could influence cell cycle through its impact on transcription and translation activities. Cell tracking experiments have indeed revealed that mitochondrial content and cell cycle length are correlated [42]: after cell division, daughter cells with more mitochondria progressed through the cell cycle proportionately faster than their sisters.

In order to assess the simultaneous contributions of mitochondria and cell cycle to protein variability, mitochondrial content and cell cycle state can be quantified simultaneously with protein abundance in single cells. To properly separate the contributions of both factors, the effect of each factor on the other should be removed before correlating with protein abundance. These analyses show that cell-cycle contribution to protein variability, per se, is low compared to mitochondrial contribution [48]. This is in agreement with a recent work quantifying cell-cycle effects on the expression of many genes in different cell lines, that revealed only a modest contribution (5–17%) to gene expression variability [70].

## Fluctuations in cellular components

Cells in an isoclonal population may present large differences in the abundance of organelles and molecular components. Two processes can cause these differences: the dynamics of organelle/molecular biosynthesis (which may be different in each cell) and the uneven segregation of cellular components at cell division. Detailed analyses show that much of the fluctuations experimentally attributed to biochemical noise in synthesis/degradation reactions can indeed stem from partitioning errors of cellular components at cell division [71].

In HeLa cells, quantification of mitochondrial content after cell division showed that the ratio of mitochondria among daughter cells follows a skewed distribution, pointing out to an asymmetry in mitochondrial segregation [42, 46]. Moreover, each division behaves as an independent random partitioning event with no memory of previous divisions [42]. This suggests that mitochondria segregate following a binomial partitioning, similar to the separation of molecular components such as mRNA and proteins [71]. Simple models of cell dynamics with binomial partitioning of mitochondria and independent cell volume fluctuations reproduce well the observed experimental results [46], as shown in Fig. 2B.

Another possible factor causing heterogeneity in mitochondrial content is the dynamics of mitochondrial biogenesis, which is characterized by continuous cycles of fusion and fission. However, these processes are fast compared to the cell-cycle period: on average, each mitochondrion undergoes around 5 fusion:fission cycles per hour [72], and this will likely promote a "steady" population of mitochondria inside the cell [72, 73]. It is interesting to note that mitochondrial fusion and fission dynamics are regulated during the cell cycle [74], with mitochondrial fission enhanced during mitosis, which could facilitate passive and stochastic segregation of mitochondria to daughter cells. This mechanism is not universal: some cells undertake asymmetric segregation of mitochondria due to active selection mechanisms, which could act as a quality control [75]. Recently, it has been demonstrated that aged mitochondria are asymmetrically partitioned in mammalian epithelial stem-like cells, and cells with fewer old mitochondria maintained stemness properties [76].

Another level of complexity is introduced by *intracellular* heterogeneity in mitochondrial organization and function [77]: many studies in different cell types have revealed a large variability in subcellular localization, morphology, and membrane potential of mitochondria, probably regulated by mitochondrial dynamics and autophagy [78]. This intracellular heterogeneity has physiological consequences: for instance, fused mitochondrial states induce effects like increased energy production, protection against apoptotic signals, or changes in cell proliferation [79]. Finally, the proportion of mutant mitochondrial DNA in a cell (heteroplasmy) can be also highly variable between cells and across tissues [80]. Differences in heteroplasmy may drive abrupt changes in transcriptional programs [81]. Possible sources of mitochondrial heterogeneity and its potential impact on gene expression and cell-to-cell variability are summarized in Table 1.

All these studies underscore the need for more detailed analyses of the contribution of mitochondrial heterogeneity, in function and mutational load, to individual gene expression profiles. This could be achieved with experiments where single-cell transcriptomics is combined with accurate quantification of ATP, ROS, and other metabolites in each cell.

**Table 1. Sources of mitochondrial heterogeneity and its impact on gene expression**

| Mitochondrial parameter | Origin | Type of variability | Mitochondrial products | Effect on gene expression |
|---|---|---|---|---|
| Mass | Asymmetric partitioning at cell division [42, 46]<br>Cell-cycle stage [48]<br>Mitophagy [73] | Non-genetic cell-to-cell variability | ATP<br><br>Acetyl-CoA<br>SAM<br>NAD+<br>ROS | Chromatin remodeling [56], transcription elongation [42], translation [42]<br>Histone acetylation [58]<br>Histone and DNA metylation [58]<br>Histone deacetylation [123]<br>Histone H3 glutathionylation [124], transcription factor activation/repression [125] |
| Structure | Fusion/fission events [79]<br>Cell-cycle stage [74] | Non-genetic cell-to-cell variability | ATP [126], ROS, calcium [73] | Chromatin remodeling, transcription elongation and translation. Transcription factor activation/repression (signaling, proliferation, apoptosis [79, 127]) |
| Functionality | Changes in membrane potential [77], mitochondrial dynamics [78] | Non-genetic cell-to-cell variability | ATP, ROS, calcium [78] | Chromatin remodeling, transcription elongation, translation. Transcription factor activation/repression (signaling, cell homeostasis, apoptosis [77]) |
| Heteroplasmy (mtDNA mutations) | Asymmetric partitioning at cell division, random mtDNA turnover [45, 128] | Genetic cell-to-cell variability | ATP, ROS [129] | Chromatin remodeling, transcription elongation, translation. Transcription factor activation/repression through retrograde signaling [81, 125] |

Here, we summarize several mitochondrial parameters that can be heterogeneous from cell-to-cell in an isoclonal population, the possible origin of this heterogeneity, the mitochondrial products that can be variable and its effect on gene expression.

## Global constraints and scaling of gene expression

Cells respond to changes in external conditions by altering the expression of thousands of genes [82]. While some genes are specifically regulated by the shift in condition, most of the response is non-specific, suggesting that it may be the consequence of changes in cellular factors affecting many genes. Accurate genome-wide measurements of promoter activities in yeast and *E. coli* with fluorescent reporters, across 10 different growth conditions, revealed that 60–90% of promoters change their expression between conditions by a global scaling factor [83] that depends only on the growth condition.

We have seen that cells with more mitochondria possess more biosynthetic capabilities, and it is then likely that pronounced differences in mitochondrial content cause a change in the expression activity of many genes. To investigate this possibility, we sorted a clonal population of HeLa cells into two subpopulations with around fivefold changes in mitochondrial mass (low and high conditions) (Fig. 3A), and then sequenced RNA extracted from both populations. We show in Fig. 3B the expression activity (mRNAs) of around 13,000 genes in low versus high condition (~half of the human genome). There is a clear scaling trend, with a scaling factor ~2.3 between cells with low and high mitochondrial content.

A functional analysis showed that genes involved in macromolecular homeostasis (synthesis and degradation of mRNA and proteins) were among the most affected by mitochondrial levels. Within this group, we found that mitochondrial content has a special impact on genes coding for ribosomal proteins (Fig. 3B). It is tempting to speculate that energy is mediating the redistribution of cellular resources [36]: cells with a large energy budget are capable of increasing the levels of their proteins, but devote part of this "excess" energy to the synthesis of ribosomes. This increased ribosomal biosynthesis can be also thought as a way to store the excess energy [84].

An interesting question is if more energetic cells, apart from increasing the production of mRNAs and proteins, increase also the variability (as measured by the CV). We think that this is probably not the case: quantifying the global transcriptional activity in control HeLa cells and in cells with ATP depleted, we find the same coefficient of variation (Fig. 3C and D). This means that cells with more mitochondria increase the average amount of mRNA, but also the deviations around this average at the population level. This happens also at the single cell level: when analyzing foci of transcriptional activity inside a cell, we find that cells with differences in mitochondrial content display the same CV regardless average activity (Fig. 3E–G). This suggests that cells are able to adapt their global gene expression activity to their energy budget.

## More is different: Qualitative alteration of the transcriptome through alternative splicing

So far, the general picture we gave is that mitochondria would play the role of a cellular "volume knob" to amplify or decrease gene activity, with mitochondrial content linearly modulating relevant rate parameters of the gene expression cycle. Although on a global scale (measuring average amounts of transcripts, proteins, histone modifications, etc.), it seems to be the case, we can guess that, when considering specific genes or looking at molecular processes in more detail, there can be many sources of non-linearity in response to changes in mitochondrial levels. One example is shown in Box 1: the reactions sketched in the basic "Central Dogma" model in Fig. 2 are in fact composed of different molecular steps, coupled in many

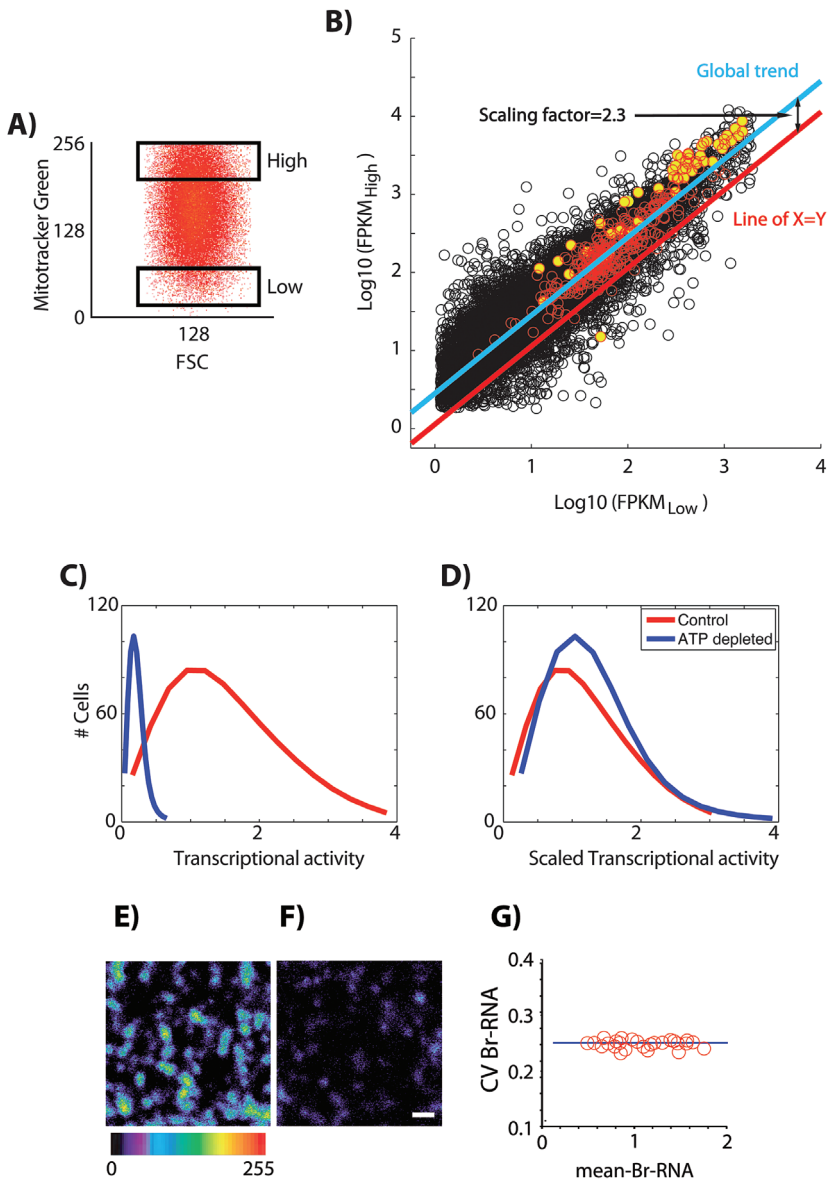



**Figure 3.** Global effects of mitochondrial levels on gene expression. **A:** HeLa cells are sorted according to their mitochondrial content (as reported by MG) into two subpopulations: low and high. **B:** RNA-seq analysis of the two subpopulations. Expression data in fragments per kilobase of exon per million (FPKM) are corrected by the amount of mRNA per cell in each sample, to get comparable transcript amounts between samples. We show, for genes expressed above the detection limit, the expression levels in low and high samples (black circles) in log–log scale. Red line represents the case in which expression in low and high is the same. The blue line represents the global trend of the data using robust linear regression [83], which shows a scaling factor of ~2.3 (implying that on average genes double their expression in the high sample). We have plotted with red symbols the genes that are involved in ribosome biogenesis [122]. From those, the genes coding for ribosomal proteins are colored in yellow. **C:** Distribution of transcriptional activity (as measured by the amount of the immediate transcription precursor BrU) in two clonal cell populations: control HeLa cells (red line) and HeLa cells with ATP depleted by treatment with Azide (blue line). **D:** The same as in C, but the transcriptional activity has been scaled by its average value in both populations. **E** and **F:** High power images of transcription foci inside the cell nucleus of two cells. They show that all the transcription foci inside the nucleus have consistently either high elongation rate (E) or low elongation rate (F). **G:** The analysis of the coefficient of variation (CV) of fluorescence intensities inside the nucleus for different cells shows that although the mean intensity changes, the CV remains constant.

cases (for instance, histone acetylation accelerates the transition of RNAP from initiation to elongation [85]). Many of these steps can have rates that depend on ATP, but differently. When combining these rates into "effective" constants, the result may be a non-linear function of ATP (Box 1).

A more drastic example is the effect of mitochondria on alternative splicing [48]. Alternative splicing (AS), the process by which primary transcripts (pre-mRNAs) are spliced into different isoforms, increases dramatically proteome diversity: it has been estimated that the human genome encodes more than 100,000 proteins with different primary sequence from the approximately 23,000 protein coding genes [86]. It is known that the number and relative abundance of mRNA isoforms can be highly variable [87], suggesting that much of the AS may be a consequence of fluctuations in the splicing machinery [88, 89]. Single-cell transcriptomics in immune cells has recently revealed a large heterogeneity in alternative splicing at the individual cell level [90].

Analysis of the transcriptome in the low and high subpopulations showed drastic alterations in the relative abundance of the RNA isoforms [48]. Interestingly, we found that a noticeable fraction (~40%) of the differentially expressed isoforms corresponded to inversions of abundance between the two samples (one isoform more expressed in high and the other in low, Fig. 4A and B). These inversions are likely due to large changes in splice site choice mediated by mitochondrial levels [48]. How can AS be altered in such a way by mitochondria? This is probably the combined action of many factors: for instance, mitochondrial differences entail changes in elongation speed, and transcription elongation may be coupled to AS [91] (Fig. 4C and D). Chromatin remodeling, where energy plays such a critical role, also influences the outcome of AS, both by distinguishing exon and intron recognition and by regulating the recruitment of specific splicing factors [92].

## Consequences of mitochondrial cell-to-cell variability

### Mitochondria and physiological processes that require gene activation

The broad access to omics techniques is changing our view of the interplay between

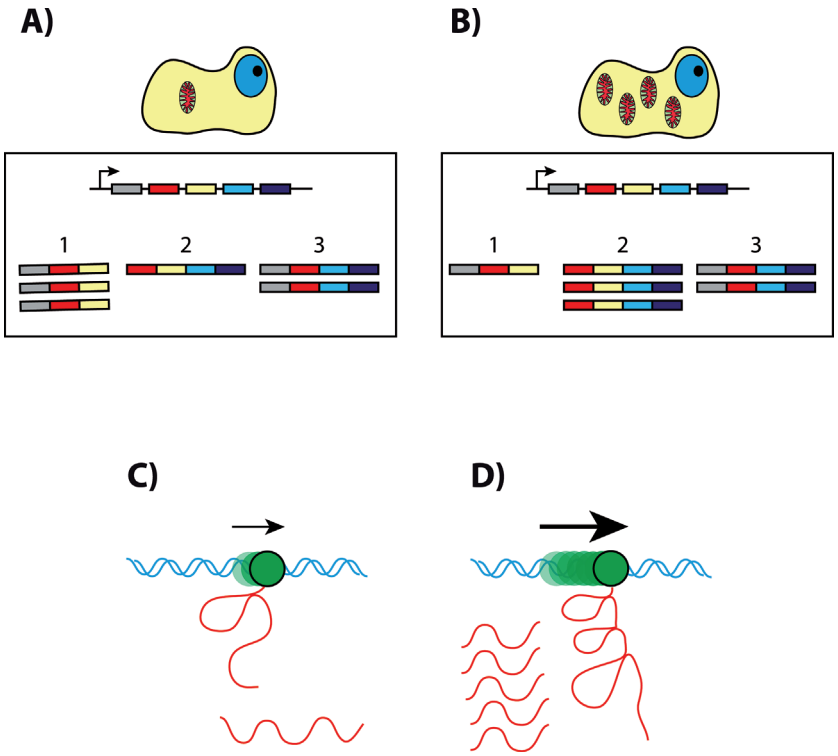



**Figure 4.** Effect of mitochondria dosage on alternative splicing. Cells with different amounts of mitochondria show different frequencies of mRNA isoforms. As an illustration, a gene with the same amount of transcripts in cells with low (**A**) and high (**B**) mitochondrial content may display a large variability in the alternatively spliced mRNAs. Isoforms 1 and 2 show an opposite expression pattern: isoform 1 is more abundant in the low type, while isoform 2 is more abundant in the high. **C** and **D:** RNA Pol II elongation speed influences the RNA secondary structure, which is a key determinant of alternative spliced form choice.

metabolism and gene expression. For instance, it is usually accepted that aberrant gene expression in tumor cells originates a metabolic reprogramming. The current understanding is that metabolic alterations also entail genome-wide changes in the transcriptional program. Thus, any process that involves a shift in cellular energy will likely result in a global gene expression change.

One paradigmatic example is the immune response, where different cell types react to antigens on an individual basis that involves dramatic changes in gene expression. There exists a big body of literature on the function of energy metabolism during the immune response [93, 94], and relating metabolic reprogramming mediated by mitochondria to the activation of immune cells [95]. The main conclusion from these studies is that the metabolic reprogramming is needed to achieve the correct physiological function of immune cells, as well as an essential requirement for cell activation, which depends on the expression of surface costimulatory molecules and the secretion of cytokines and chemotactic factors. Moreover, ATP is used to support the massive increase in biomass needed for cellular expansion [96]. These evidences suggest that the metabolic reprogramming may precede gene expression rather than being its consequence. If this is true, mitochondria should play a role in early gene activation. Kinetic analysis of T lymphocytes activation have shown that both hydrogen peroxide and superoxide anion are generated within 15 min of TCR cross-linking [97], possibly from activated mitochondria [98]. In the future, careful metabolic analyses at very short time after activation will be needed to prove the connection with early gene expression.

It is not intuitive, how a general mechanism (metabolism) can drive individual cells to express very well defined genetic programs. A possible explanation is that when metabolic reprogramming takes place, chromatin opens and the transcription factors can reach the regulatory elements of their target genes. Since cells express heterogeneous amounts of specific transcription factors, once the chromatin is open, the initial levels of those will determine the individual transcription program. Is this mechanism feasible? Some clues come from what we have learnt in the reprogramming of somatic cells (iPSCs). The success of the iPSCs technique resides in the fact that the cell to be reprogrammed must express a particular set of transcription factors (Oct4, Sox2, cMyc, and Klf4). However, the efficiency of the original technique was very low, around 0.1% for mouse fibroblasts [99] and 0.01% for human fibroblasts [100]. To overcome low yield, specific molecules acting on the epigenetic machinery have been used: one example is valproic acid (VPA) that inhibits the histone deacetylase HDAC, keeping chromatin open and increasing the efficiency of the process by 100 times [101]. Recent results suggest key roles of mitochondria and metabolism in the maintenance of pluripotency, differentiation, and reprogramming of induced pluripotent stem cells (iPSCs) [102, 103].

If energy redistribution is preceding gene expression changes, then some cell signaling must be mediated by mitochondria. We have several evidences of this: one is glucocorticoid signaling, in which a non-canonical activation pathway involves the translocation of the complex glucocorticoid/receptor to the mitochondria, modulating its function [104]. Another example is the Notch pathway regulating cell proliferation, differentiation, and death in all metazoans. In the non-canonical pathway (which controls the maintenance of human cancer stem cells [105]), Notch interacts with PTEN-induced kinase 1 (PINK1) modulating mitochondrial function, possibly raising ATP levels. Thyroid hormones are signaling molecules with two different effects on mitochondria: the first is a rapid stimulation of respiration, which is evident within minutes/hours after hormone treatment. The second response is more sustained and occurs in the range from one to several days after hormone treatment, and leads to mitochondrial biogenesis and to a change in mitochondrial mass [106]. These examples suggest that signaling mediated by receptors consists of a fast response, probably involving changes in mitochondrial function (inducing ATP increase),

followed by more sustained changes in the expression of the targeted genes.

### Mitochondria and pathology

Another consequence of the regulatory effects of mitochondria on gene activity is that the malfunction of mitochondria will result in aberrant gene expression. In such a way dysfunctional mitochondria appear as a common factor in the pathophysiology of many diseases [107]. For instance, cells from individuals with different pathologies that carry mutations in the mitochondrial DNA (mtDNA) show also aberrant expression of many nuclear genes [81].

We have discussed how mitochondria can modulate AS: then, mitochondrial dysfunction may result in the alteration of AS. Aberrant AS is very common in human pathology: around 50% of human genetic diseases arise from mutations that affect the AS process [108]. If aberrant AS is caused by mitochondrial dysfunction, this effect should be more prominent in tissues with a high dependence on ATP. We find many evidences in support of this in cardiac and brain diseases.

AS is largely affected in heart diseases [109]. In this context, genes with altered AS are related with the sarcomere, ion channels, and signaling molecules [110]. Some of the anomalous AS events in cardiac genes are associated with mutations in the splicing site. Mitochondria dysfunction has also been associated with many heart pathologies including hypertrophic and dilated cardiomyopathy, sudden death, ischemic cardiomyopathy, myocarditis, and aging [111], which not surprisingly show aberrant AS. These diseases evolve to heart failure (HF). HF is a complex process with different physiologies, but all of them have in common a decrease in ATP (up to 40%) and in phosphocreatine [112]. Mitochondrial dysfunction appears invariably as an early event in HF [113]. Moreover, therapies that improve mitochondrial function seem to improve the long-term survival of patients with chronic HF [114]. In neurodegenerative diseases, such as Alzheimer and Parkinson, and neurodevelopmental diseases like autism, aberrant AS has been documented [115]. These diseases are also associated with dysfunctional mitochondria [116, 117], and are ameliorated by treatments designed to improve mitochondrial function [117].

Cancer is another paradigmatic example where metabolic reprogramming mediated by mitochondria takes place. Animal cells need growth factors to direct nutrient uptake. In environments with limiting levels of growth factors, differentiated cells adopt a catabolic metabolism mainly based on oxidative phosphorylation by mitochondria to maximize the efficiency of ATP yield. In contrast, when growth factors are abundant, cells increase their nutrient uptake and adopt an anabolic metabolism favoring biomass production. In pathological situations, like cancer, growth-factor signaling can be permanently activated, and the metabolism may be reprogrammed to a glycolytic phenotype [14]. Interestingly, recent findings suggest that in many cancer types mitochondria are also reprogrammed for macromolecular biosynthetic activity [118]. The metabolic reprogramming of solid tumor cells seems to be also important for dissemination: tumor circulating cells show an enhanced ATP respiration rate and oxygen consumption compared to primary tumor cells [119]. Aberrant AS is also a hallmark of cancer cells [120], thus it is tempting to speculate that alteration of mitochondrial function in cancer is also linked to abnormal AS.

## Conclusions and perspectives

Heterogeneity in gene expression and in the amounts of the cell's molecular components and organelles can be an important source of phenotypic variation. By far, among all cellular organelles, mitochondrion has made possible life as it is: by an enormous increase of the cell's energy power, by its role on cellular signaling, differentiation and death, and through its influence on transcriptional complexity.

In this review, we focused on the role played by mitochondrial content on cell individuality, mainly through its impact on the different steps of the gene expression cycle due to variable ATP availability. In this respect, experimental evidences are just starting to accumulate, and more work is needed to understand the molecular mechanisms connecting mitochondrial heterogeneity to specific processes, such as chromatin remodeling or alternative splicing. Moreover, we have discussed in detail the influence of a single parameter, namely the amount of mitochondrial mass, which is proportional to ATP. Structural and morphological variabilities may also be important. Different mitochondrial states are known to influence several cellular processes, including ATP production. Further studies are needed to address how changes in mitochondrial dynamics and functionality modulate gene expression, as well as to assess if mitochondrial heterogeneity is equally important in different cell lines. Apart from ATP, mitochondrial respiration generates as by-products ROS and NAD+, which are known signaling molecules that could also play a role in globally modulating gene expression (Table 1).

Heterogeneity in mitochondrial mass and functionality can have far reaching consequences beyond promoting variability in gene expression, notably through its role in maintenance of calcium homeostasis, redox regulation, and apoptosis. More recently, mitochondria have been also linked to cell fate determination and development. The appreciation of the contribution of mitochondria to the genotype–phenotype relationship needs to be taken into account in strategies aimed to manipulate complex phenotypes or in personalized medicine.

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