Received: 17 April 2018 | Revised: 16 May 2018 | Accepted: 30 May 2018

DOI: 10.1111/dom.13390

WILEY

REVIEW ARTICLE

Single molecule approaches for studying gene regulation in metabolic tissues

Lydia Farack | Adi Egozi | Shalev Itzkovitz

Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel

Correspondence
Shalev Itzkovitz, Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel.
Email: shalev.itzkovitz@weizmann.ac.il

Funding information
EMBO Young Investigator Program and the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013), Grant/Award Number: 335122; I- CORE program of the Planning and Budgeting Committee and the Israel Science Foundation , Grant/Award Number: 1902/ 12 and 1796/12; Israel Science Foundation, Grant/Award Numbers: 1486/16, 1796/12, 1902/12; the Henry Chanoch Krenter Institute for Biomedical Imaging and Genomics; Howard Hughes Medical Institute (HHMI) international research scholar award; Bert L. and N. Kuggie Vallee Foundation; European Union's Seventh Framework Programme, Grant/Award Number: FP7/2007-2013; European Research Council, Grant/Award Number: 335122; EMBO Young Investigator Program; I- CORE program of the Planning and Budgeting Committee; The Lord Sieff of Brimpton Memorial Fund; Cymerman-Jakubskind Prize; Richard Jakubskind Laboratory of Systems Biology; The Leir Charitable Foundations; Henry Chanoch Krenter Institute for Biomedical Imaging and Genomics

Gene expression in metabolic tissues can be regulated at multiple levels, ranging from the control of promoter accessibilities, transcription rates, mRNA degradation rates and mRNA localization. Modulating these processes can differentially affect important performance criteria of cells. These include precision, cellular economy, rapid response and maintenance of DNA integrity. In this review we will describe how distinct strategies of gene regulation impact the trade-offs between the cells' performance criteria. We will highlight tools based on single molecule visualization of transcripts that can be used to measure promoter states, transcription rates and mRNA degradation rates in intact tissues. These approaches revealed surprising recurrent patterns in mammalian tissues, that include transcriptional bursting, nuclear retention of mRNA, and coordination of mRNA lifetimes to facilitate rapid adaptation to changing metabolic inputs. The ability to characterize gene expression at the single molecule level can uncover the design principles of gene regulation in metabolic tissues such as the liver and the pancreas.

KEYWORDS

bursty transcription, gene expression noise, mRNA degradation, single molecule fluorescence in situ hybridization, transcription rate

1 | INTRODUCTION

A fundamental feature of the mammalian body is the strict maintenance of homeostatic blood glucose levels. These levels are maintained at a tight range of around 100 mg/dL for human, in spite of large fluctuations in the amounts of glucose ingested during meals and consumed by our peripheral tissues during times of physical activity.[1] Homeostatic glucose control is a result of the coordinated actions of the endocrine pancreas and of the liver. The endocrine pancreas acts as the "metabolic brain," sensing blood glucose levels and correspondingly releasing large quantities of insulin and glucagon in times of food intake or fasting, respectively. The liver, which has been termed the "glucostat" of our body is the main producer of glucose during fasting states, and responds to these pancreatic hormonal signals by rapidly switching between glucose consumption and production in response to ingested meals and activity. While metabolic homeostasis is clearly controlled by additional organs-the brain, fat and muscles, in this review we will focus on the pancreatic endocrine cells and on hepatocytes. We will discuss distinct transcriptional strategies employed by these cells that enable them to produce sufficient

Lydia Farack and Adi Egozi have contributed equally to this work.

amounts of mRNA at the right time with high precision and minimal variability, in spite of challenging constraints. While homeostatic control involves multiple regulatory tiers, including a wide array of post-transcriptional processes, we will focus on the processes that involve mRNA production and degradation rates, amounts and intra-cellular localization. Technological advances that enable quantitative measurements of mRNA in intact tissues have facilitated studying these processes of transcriptional control in tissue cells at unprecedented sensitivity and spatial resolution.

2 | METABOLIC TISSUES ARE HETEROGENEOUS IN THEIR CELLULAR COMPOSITION

Tissues are composed of heterogeneous pools of cells that usually operate in highly structured microenvironments.[2,3] Each tissue is composed of parenchymal cells that perform the main tissue tasks, for example, the endocrine cells in the pancreatic islets of Langerhans, the hepatocytes in the liver or the enterocytes in the intestine. These parenchymal cells interact with non-parenchymal cells that support tissue functions and include endothelial cells, pericytes, fibroblasts, tissue resident macrophages and other immune cells. Understanding the interactions between these different cell types is crucial in order to dissect tissue function. This high diversity of cell types raises the importance of using single cell approaches to analyze biological processes in tissues, as opposed to bulk analyses, which average out this diversity.[4,5]

The large milieu of distinct cell types that make up our organs is not the only source of cellular heterogeneity. Even when considering a seemingly uniform population of parenchymal cells, there remains a high degree of molecular diversity related to the locations of cells in tissues and their interactions with local niches. In the liver, hepatocytes operate in polarized "lobules," in which centripetal blood flow creates gradients of oxygen, nutrients and hormones.[6–8] Indeed, it has been shown that around 50% of the hepatocyte genes are expressed non-uniformly along the liver lobule radial axis, with a spatial division of labour that seems to allocate distinct functions to the more suitable lobule microenvironments.[9] For example liver energy-demanding biosynthetic processes that include protein secretion and gluconeogenesis are allocated to the lobule regions that receive more oxygen. The islets of Langerhans also exhibit several sources of spatial heterogeneity related to the position of cells relative to blood vessels, the paracrine interactions with other cells types and the distances from the islet periphery.[10] These spatial sources of variations can potentially give rise to distinct sub-populations with specific allocation of function to distinct islet niches.[11–17] Indeed, single cell RNA sequencing highlighted diversity in gene expression among individual islet cells.[18–22] In addition to these "extrinsic" sources of variations, related to spatial locations and cell-cell contacts, tissue cells may be intrinsically different at the level of epigenetic features such as methylation patterns, chromatin states, 3D chromosomal conformations and more.

3 | SINGLE MOLECULE APPROACHES FOR QUANTIFYING GENE REGULATION IN INTACT MAMMALIAN TISSUES

The multiple sources of heterogeneity in tissues highlight the importance of using quantitative tools to measure gene expression in single cells in the intact tissue. Single molecule fluorescence in situ hybridization (smFISH) has emerged as a powerful approach to achieve this goal.[23–25] In an intact tissue, smFISH enables visualizing individual mRNA molecules of any gene of interest using libraries of singly labelled DNA sequences, or "probes," that are designed to be complementary to sequential parts of the mRNA of interest.[24] Through the local accumulation of multiple probes, individual mRNA molecules are revealed as diffraction limited spots of light under a fluorescence microscope (Figure 1A). Moreover, by designing probes against the introns of genes of interest one can visualize and quantitatively infer the dynamic processes of transcription and mRNA degradation.[26,27] Since introns are generally spliced and degraded co-transcriptionally, intact intronic segments are expected to appear only at nascent mRNAs that reside at the active transcription sites on the DNA (TS, Figure 1B). These can then be readily identified and quantified to infer the average accessibility of promoters of interest, the average RNA polymerase (RNAP) coverage for a gene, and as a result the average transcription rate. With additional measurements of the total numbers of transcripts in a cell one can also infer the average mRNA degradation rates. This approach has been applied to diverse mammalian tissues such as the liver and the intestine,[26,27] and lately also in the intact pancreas, a tissue traditionally considered highly challenging for in situ studies (Figure 1, Farack et al., submitted). Since smFISH is a spatially resolved method it also has the power to identify the location of transcripts within the cells. This can reveal patterns of intra-cellular spatial heterogeneity that can affect translation, such as nuclear retention or basal-apical mRNA polarization.[28]

The ability to interrogate gene expression in single cells at the single molecule level in intact tissues opens avenues to explore the design principles of gene regulation in tissues. In the next sections we will describe the trade-offs associated with different gene expression strategies, and examples of the use of smFISH to characterize them in the pancreas and liver.

> "Single molecule approaches are emerging as powerful tools to interrogate the processes of transcription, mRNA degradation, intra-cellular localization and translational control in single cells within intact tissues."

4 | PERFORMANCE TRADE-OFFS GOVERN THE BALANCE BETWEEN TRANSCRIPTION AND mRNA DEGRADATION

Cellular amounts of mRNA are a result of the balance between transcription rates and mRNA degradation rates. These rates span several orders of magnitude for different genes and different cell types.[29,30] To understand the trade-offs entailed in different gene expression strategies let us consider two hypothetical genes, the transcripts of which

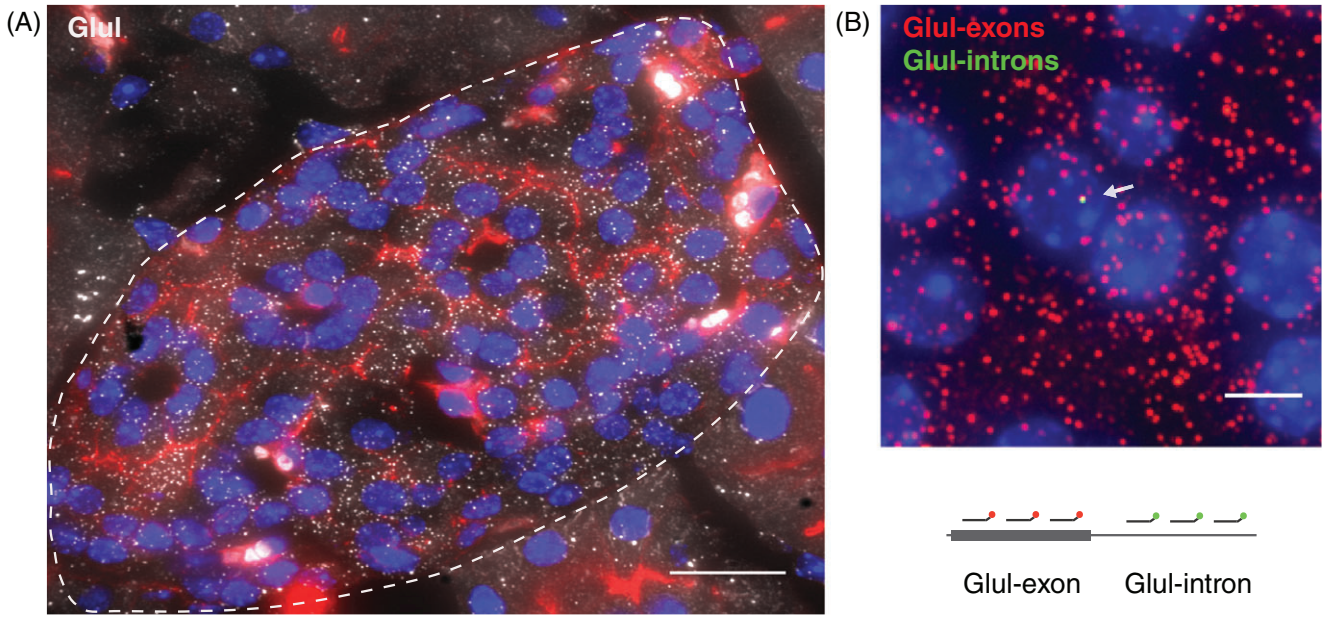

FIGURE 1 smFISH enables visualizing mature and nascent transcripts in intact tissues. A, An islet of Langerhans in the intact mouse pancreas stained with smFISH probe library complementary to the mRNA of Glul, which is encoding the enzyme glutamate-ammonia ligase (glutamine synthetase, gray dots). DAPI-stained nuclei are in blue and phalloidin in red marks the cell membranes. Scale bar is 20 μm. B, Co-localization of exonic probes in red and intronic probes in green reveal the active transcription site of Glul, which is marked by an arrow. DAPI is in blue. Scale scale bar is 10 μm

are expressed at an average level of 5 mRNAs per cell (Figure 2A). The first gene is transcribed at an average rate of 5 mRNA molecules per hour and has relatively unstable mRNAs that are degraded at a rate of once per hour (Figure 2A.1). The second gene is transcribed at an average rate of 1 mRNA molecule per hour but has more stable transcripts that are degraded on average once every 5 hours (Figure 1A.2). Producing the second gene requires less energy compared to the "futile cycle" of transcription-degradation associated with the first gene. Thus the second strategy seems more economical in terms of cellular resources. The downside, however, is that switching to a new steady-state level would take more time for the second gene (Figure 2B). If cellular levels need to be rapidly reduced, the first gene could achieve a 2-fold decrease in less than an hour of cessation of transcription (Figure 2B, orange line), whereas for the second gene a similar decrease will take around 4 hours (Figure 2B, gray line). A similar acceleration in the response time of a gene that has high mRNA degradation rates occurs when switching transcription back on again (Figure 2B). Thus the particular rates of transcription and mRNA degradation could be governed by a trade-off between cellular economy and response time.[31] Another strategy for dynamically regulating cellular mRNA levels could entail a change in mRNA degradation rates without changing the rates of transcription (Figure 2C,D). In this case, degradation rate could be specifically increased in response to external stimuli, leading to lower cellular mRNA levels. Since the response time depends only on mRNA degradation rates (Figure 2B,D), such strategy would lead to an asymmetric response profile with rapid shut-down of cellular mRNA when degradation rate is increased, but a slow ramp-up of cellular mRNA levels when reverting to the original steady state (Figure 2D).

This economy-response time trade-off is exemplified by the expression of liver gluconeogenic genes. Using smFISH techniques, Bahar Halpern et al measured the average transcription rates and mRNA degradation rates for different genes in the intact liver.[26] The genes G6pc, encoding the enzyme glucose-6-phosphatase, and Pck1, encoding the enzyme phosphoenolpyruvate carboxykinase 1, exhibited intense and frequent transcription sites in a fasted state, with around 30 RNAPs engaged in transcription for each gene copy. The estimated lifetimes of the mRNAs for these genes were around 20 to 30 minutes. These mRNA lifetimes are significantly shorter than the median reported lifetime of 9 hours for mammalian transcripts.[30] Consistent with these excessively short mRNA lifetimes, cellular mRNA levels declined by more than 3-fold within 60 minutes of re-feeding of fasted mice, when transcription of these genes abruptly stopped.[26] A similar decrease was also seen at the protein levels.[26] One of the main functions of the liver is to act as a "glucostat," constantly regulating blood glucose levels in face of external perturbations such as meals and exercise. Shutting down these 2 main enzymes responsible for hepatic glucose output therefore seems important to avoid excessive increase in blood glucose levels following a meal. Enzymatic activities can be regulated at post-translational levels, for example, by protein modifications such as phosphorylation. It is interesting that in the liver, gene regulation for these important enzmyes is performed at the very basic level of transcription and mRNA degradation.

> "mRNA degradation rates dictate the response time of cellular mRNA levels."

5 | BURSTY TRANSCRIPTION, THE FIDELITY-NOISE AND RESPONSE-NOISE TRADE-OFFS

Our picture of mRNA production considered a single rate that modulates the amounts of actively transcribed mRNA. Studies in a range of

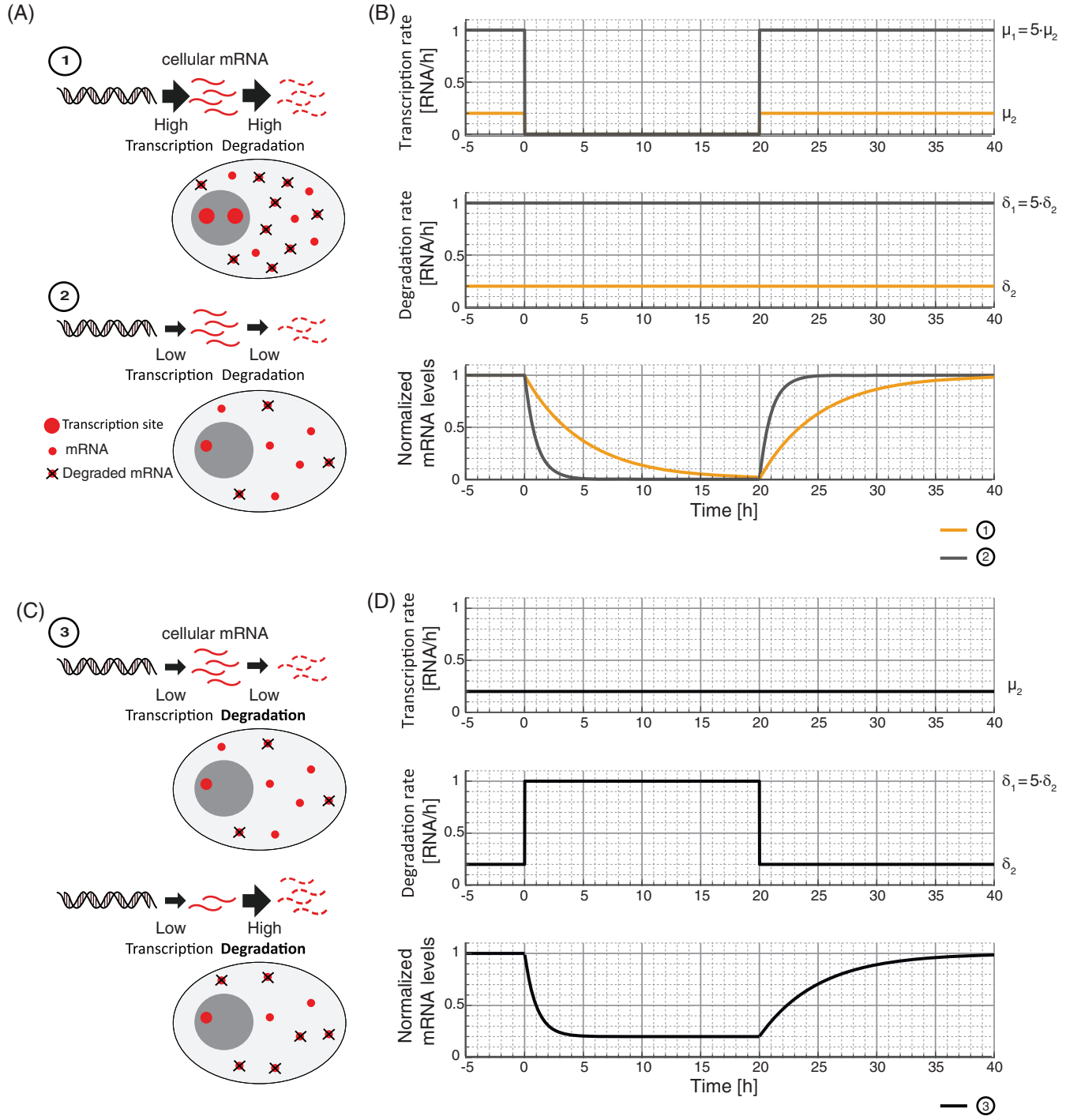

FIGURE 2 Degradation rates of mRNA govern response times to changes in transcription. A, A cartoon representing two hypothetical genes with similar numbers of mature mRNA molecules but different transcription and degradation rates. These rates can be measured in smFISH experiments. Gene (1), which is transcribed at a high rate, will exhibit intense fluorescent transcription sites (TS) due to the local accumulation of fluorophores on nascent mRNA that are attached to advancing RNA polymerase molecules. Gene (2), which is transcribed at a lower rate but produces more stable mRNAs will exhibit less intense TS but similar numbers of mRNA molecules. B, Transcript degradation rates (δ) determine the response time of mature cellular mRNA to changes in transcription rate (μ). High rates facilitate a rapid response (1, orange curve), whereas low rates lead to a slow response (2, gray curve) when the transcription turns off or on. C, Switching between steady states through modulation of mRNA degradation rates leads to an asymmetric response. Shown is a hypothetical gene (3) for which the degradation rate changes from low to high levels. D, since response time is governed by mRNA degradation rates, the transitions between steady states are asymmetric—fast response when degradation rate is high, slow response when degradation rate is low

organisms, from bacteria, through yeast and mammals demonstrated that transcription of many genes is not a continuous process but is rather stochastic, leading to punctuated production of transcripts.[26,32–37] These transcripts seem to be produced non-uniformly over time in transcriptional "bursts" (Figure 3). The precise biochemical nature of these bursts is not clear, but could entail dynamic shifts of the chromatin between a nucleosome wrapped "closed" state and an "open" state, governing the promoter

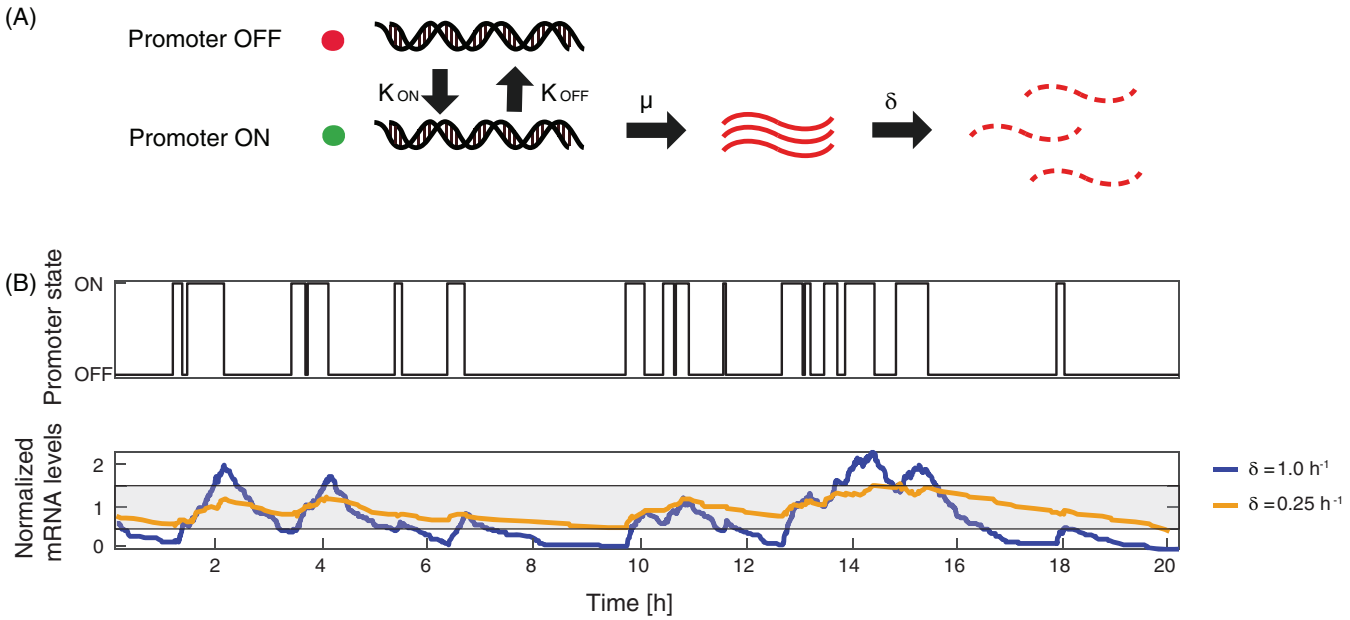

FIGURE 3 Bursty transcription can generate temporal fluctuations in mRNA content, also termed "gene expression noise". A, Diagram of a 2-state model of bursty transcription with a promoter opening rate K_{on}, promoter closing rate K_{off}, transcription rate μ and mRNA degradation rate δ. B, Gillespie simulation of mRNA cellular content under a model with unstable mRNA having a lifetime on the order of magnitude of the burst periods (blue, degradation rate $\delta = 1\ h^{-1}$) and stable mRNA with a mRNA lifetime that is longer than the typical inter-burst periods (orange, degradation rate $\delta = 0.25\ h^{-1}$). Top shows promoter states, simulated according to a bursty model with $K_{on} = 1\ h^{-1}$ and $K_{off} = 3\ h^{-1}$. When the promoter is active, the transcription rate is $\mu = 80$mRNA/h for the blue plot, $\mu = 20$mRNA/h for the orange plot, to maintain the same steady state average mRNA content between the two compared scenarios. Expression is normalized by the mean, gray patch highlights the region in which mRNA levels are within 0.5–1.5 of the mean (coefficient of variation below 0.5)

accessibility to activating transcription factors.[33] Under this picture of bursty transcription the rate of mRNA production now depends on two independent parameters-the "burst frequency" and the "burst size." The burst frequency is the rate of switching between active and inactive states and governs the fraction of time a gene is in the transcriptionally active state. The rate of RNAP recruitment during an active state is given by the "burst size." Both of these parameters can be measured in situ using smFISH. The burst frequency correlates with the fraction of DNA loci, that are actively transcribed at any given moment, whereas the RNAP occupancies, extracted from the intensity of the nuclear TS dots, correlate with the burst size.[26,27]

What could be the advantages of producing transcripts in intermittent bursts? One could be the protection of DNA from mutagenic agents. DNA in a closed conformation is inaccessible to such potential insults and therefore genomic fidelity is increased.[38] Another advantage could be the compact packaging of DNA, thus clearing up the congested nuclear volume for diffusion of transcripts and transcription factors.[39] A potential disadvantage of bursty transcription is that it can generate temporal fluctuations in the mRNA content of a cell. During the periods at which the promoter is inaccessible, mRNA degradation reduces the amounts of cellular mRNA, whereas the cellular mRNA levels increase during an open state (Figure 3B). These temporal fluctuations in mRNA content that are caused by the stochastic nature of bursty transcription have been termed "gene expression noise."[33,34] They can generate profound variations in the cellular state of a given cell over time (Figure 3B) and differences in cellular states of similar cells that sense the same environment. Such variability could be advantageous in some contexts, for example, to facilitate diverse responses to environmental challenges.[34] However, when cellular function depends on a fine-tuned level of expression, as it is often the case in metabolic tissues, intrinsic variability could potentially reduce overall performance.[26]

The impact of bursty transcription on the amount of noise can be minimized when mRNA lifetimes are long. During the stochastic periods between bursts, when a gene is transcriptionally silent, mRNA levels decline as transcripts are degraded. A low mRNA degradation rate leading to a long mRNA lifetime effectively buffers the noise created by bursts, as mRNA levels barely go down before another burst occurs (Figure 3B).

In the mammalian liver, burst fractions have been quantified for several genes. Notably, bursty transcription and mRNA lifetime were coordinated in a way that minimizes gene expression noise. Some genes, such as Actb (Figure 4B), are transcribed in rare bursts, but have stable mRNA with a half-life of around 14 hours.[26] Others, such as the β-cell gene Acly (Figure 4A) and the liver gluconeogenic genes Pck1 and G6pc, are expressed in a non-bursty manner. Pck1 and G6pc are hepatocyte genes with short mRNA lifetimes, to facilitate rapid response to metabolic stimuli. In fasted states, when hepatocytes should provide constant glucose flux, their non-bursty mode ensures relatively homogenous and stable cellular transcript content. In summary, frequencies of transcriptional bursts and mRNA lifetimes shape the noise-response trade-off that cells face.

> "Transcription in metabolic tissues often occurs in stochastic bursts, leading to fluctuations in the cellular mRNA content."

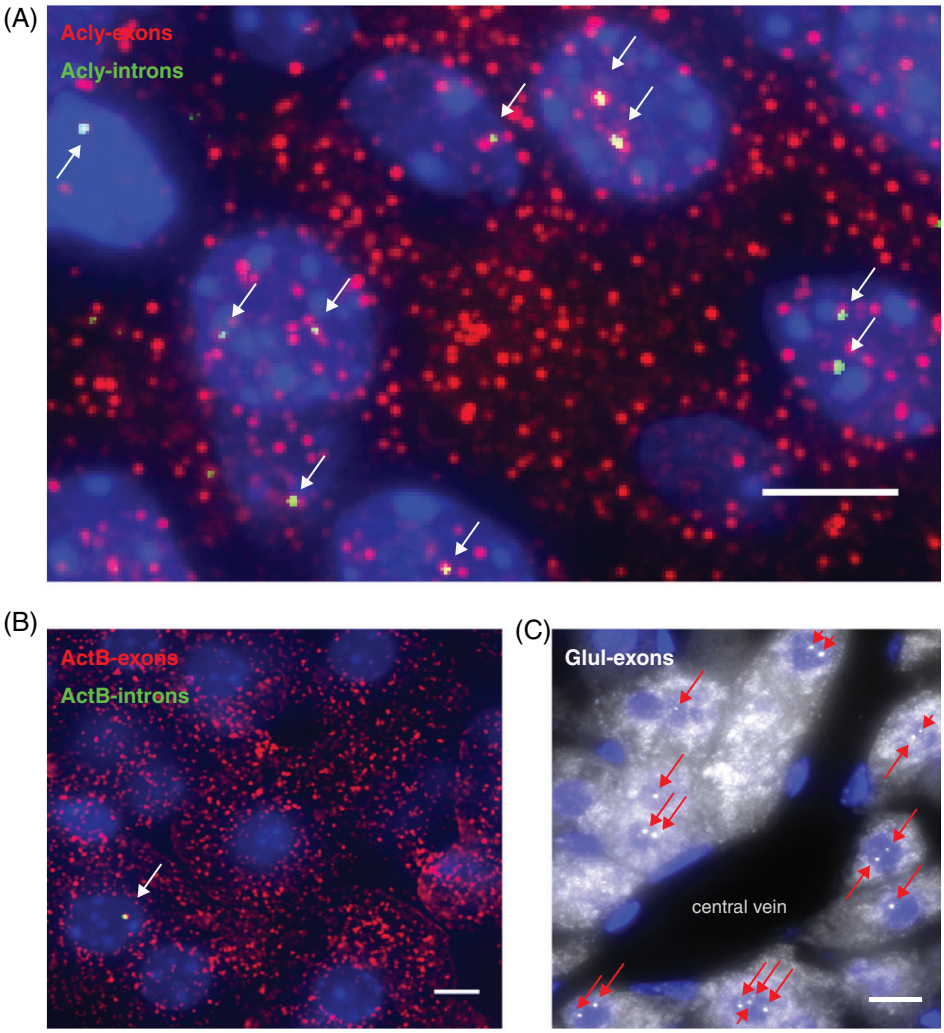

FIGURE 4 Examples of bursty and non-bursty transcription in the pancreas and liver. A, Acly mRNA is expressed in a non-bursty manner in pancreatic β-cells. Co-staining of β-cells in the intact pancreas with probes for exonic (red) and intronic (green) regions reveal non-bursty transcription sites of Acly. (B) In hepatocytes, ActB exhibits bursty expression, whereas (C) Glul is expressed in a non-bursty manner around the central vein of a liver lobule. Transcriptions sites are indicated by arrows. DAPI is in blue. Scale bar is 5 μm for (A,B) and 10 μm for (C)

6 | BURSTY TRANSCRIPTION LIMITS THE MAXIMAL TRANSCRIPTIONAL OUTPUT FROM A GENE

Producing mRNA in transcriptional bursts introduces another challenge to cells-it limits the maximal transcriptional output from a gene. To understand this limitation, let us consider a toy model of transcription where a train of RNAPs that fully covers the body of a gene of interest advances at a constant rate along the gene (Figure 5A). The affinity of the RNAP to the target promoter is so high that whenever a RNAP molecule falls off the 3′ end of the gene and the RNAP train proceeds, a new RNAP immediately binds the freed-up space at the 5′ end of the gene. At steady state, the limiting rate of mRNA production for such a gene is dictated by the footprint of the RNAP and its speed. Since the footprint of the RNAP II is around 40 bps,[40] and its speed is around 34 bps/s,[26] the maximal transcriptional output is around 0.85 mRNA per second, or 2600 mRNA per hour (Figure 5A). A bursty gene that is active only 50% of the time will reach at most 50% of this output, and generally less, due to the initiation phase of filling up the gene body with RNAPs when a burst initiates. Notably, the longer the gene, the longer it will take until the gene body is completely covered with RNAPs and until the first transcript is released. This could be substantial, as a typical gene of size 27 kbps[41,42] will exhibit an initiation phase of at least 13 minutes. Thus, cellular transcription rates of a gene expressed in a diploid mammalian cell cannot exceed a few thousands of nascent mRNAs per hour. Therefore, maintaining a high steady state of mRNA levels would require extending mRNA lifetime. As discussed above, this limits the cell's ability to rapidly switch between steady states in response to environmental conditions.

Cells in the pancreas and liver produce massive amounts of secreted proteins. The genes encoding these secreted proteins often exhibit very high levels. The β-cell seems to be the most extreme example, with more than 25% of its mRNA content represented by transcripts of insulin (Figure 5B). Indeed, the lifetime of insulin mRNA is relatively long with approximately 30 to 80 hours depending on the metabolic state.[43] Presumably, these long mRNA lifetimes facilitate achieving the required high mRNA levels for this gene. Another example for an abundantly expressed gene is Glul, which encodes the enzyme glutamate-ammonia ligase (glutamine synthetase), in hepatocytes that

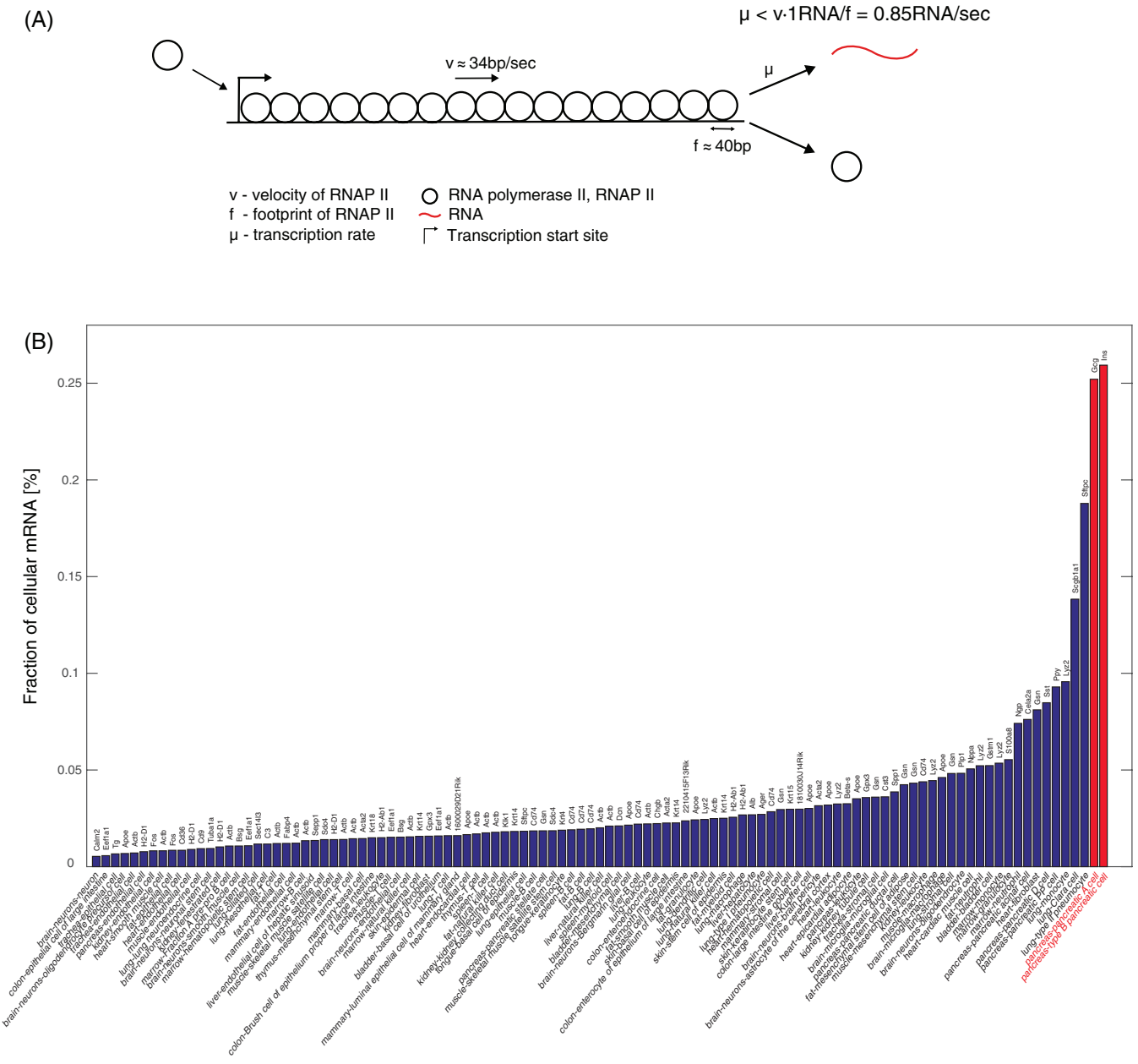

FIGURE 5 Long mRNA lifetimes are required for achieving high mRNA levels of key metabolic genes such as insulin and glucagon. A, The transcriptional output from a gene is dictated by the duration and frequency of a burst as well as by the occupancy and speed of RNAPs. A toy model of transcription with a train of RNAPs that fully covers the body of a gene of interest. The occupancy of a gene with RNAPs depends on the gene length and the footprint of the RNAP ($f \approx 40$ bp). RNAPs advance at a constant speed ($v \approx 34$ bp/s) along the gene. Notably, the longer the gene, the longer it will take until the gene body is completely covered with RNAPs and until the first transcript is released. When the gene is fully covered, transcription rate is maximal and independent of gene length. It is limited by the time to displace a single RNAP ($\mu \leq v/f = 0.85$RNA/s). When transcription rate is maximal, mRNA stability becomes the limiting factor for cellular mRNA levels. B, Insulin and glucagon take up an exceedingly high fraction of total cellular mRNA in pancreatic islet cells. These high levels require both elevated transcription rates and increased mRNA stability. Shown are the fraction of total cellular mRNA molecules allocated to the most highly expressed genes in 107 cell types from the mouse cell atlas.[4] We removed un-nucleated erythrocytes as well as cells annotated as "unknown cells." The analysis demonstrates that for most tissues the highest expressed gene contributes up to 5% of all cellular mRNAs. Notably, pancreatic β- and α-cells (red) show elevated mRNA levels of insulin and glucagon of more than 25% of all cellular mRNAs

surround the central vein of a liver lobule. This gene seems to be expressed in a non-bursty manner, in this tissue with intense TS apparent with smFISH (Figure 4C). In contrast, ActB is expressed in a bursty manner within hepatocytes, with rare TS (Figure 4B). The limited transcriptional output from genes poses an abundance-response trade-off—to achieve high steady state abundance of mRNA the cell must impose a long mRNA lifetime, limiting the ability of the gene to efficiently respond to stimuli by rapidly changing steady state levels.

> "Bursty transcription protects DNA but limits the transcriptional output and generates cell-to-cell variability"

7 | BUFFERING MECHANISMS THAT REDUCE THE VARIABILITY GENERATED BY BURSTY TRANSCRIPTION

An important requirement for cells operating in metabolic tissues may be a fine-tuned level of expression for genes, particularly the ones involved in maintaining physiological homeostasis. If this were indeed the case, we could have expected that such genes would not be expressed in noisy bursts. It, therefore, came as a surprise that in the intact mammalian liver key liver genes were found to be expressed in bursts.[26] Notably, however, several mechanisms are at play to reduce the impact of bursty transcription on cellular variability. One mechanism, which was already discussed above involves the coordination of burst frequencies and mRNA lifetimes. When mRNA lifetimes are long compared to the periods at which a promoter is in a closed state, cellular mRNA levels decline only slightly before the next burst occurs (Figure 3B, orange line). Indeed, many bursty genes in the liver have long mRNA lifetimes.[26]

A second surprising mechanism for buffering the transcriptional noise that is associated with bursty transcription is related to the intracellular localization of transcripts. The traditional view of mRNA lifetime posits a short nuclear mRNA period of a few minutes and a relatively long cytoplasmic period of hours, during which transcripts are translated and eventually degraded.[44–46] In contrast to this picture, smFISH and transcriptomics of nuclear and cytoplasmic cell fractions demonstrated that up to 14% and 30% of the protein coding genes in hepatocytes and β-cells, respectively, have more mRNA copies in the nucleus compared to the cytoplasm.[47] These nuclearly retained genes included critical genes such as Gck and Mlxipl, which are encoding the enzyme glucokinase and the transcription factor Chrebp, respectively (Figure 6B,C). Using simulations and transcript counting, it was demonstrated[47] that nuclear retention of mRNA can buffer cytoplasmic noise. While nuclear mRNA content can fluctuate in line with bursty transcription, the delay in the export of transcripts to the cytoplasm produces a constant "trickle" of mRNA from the nucleus, and therefore balances cytoplasmic mRNA levels between bursts (Figure 6A).[47–50]

Diverse mechanisms can give rise to the broad nuclear retention of mRNA in β-cells and liver. At least for some of the nuclearly retained genes, there seems to be a substantial co-localization between the nuclear mRNAs and nuclear speckles,[51] membrane-less organelles with diverse functions such as post-transcriptional splicing. Other processes could include a slow bulky diffusion through the nuclear space, or retention at the 3' UTR of the genes.

> "Elongated lifetime of mRNA and nuclear retention buffer the cytoplasmic mRNA content from burst-associated fluctuations."

8 | mRNA LOCALIZATION AND POST-TRANSCRIPTIONAL REGULATION

Nuclear retention of mRNA can potentially have another utility—facilitating an abrupt increase in cytoplasmic mRNA content in response to external stimuli. Transcription is a slow process, and de novo production of transcripts takes on average 15 minutes. In cases where rapid changes in protein content are needed one could envision a faster response if nuclearly retained mature spliced transcripts would be released into the cytoplasm in a regulated manner.[52] Thus, regulated nuclear retention can facilitate rapid translational response. Indeed, in response to glucose stimulation β-cells increase the translation of many mRNAs by around 1.3-fold and proinsulin even by up to 10-fold.[53] It will be interesting to explore whether this abrupt translational response is facilitated by regulated release of nuclearly retained transcripts.

Translational responses in metabolic tissues have also been linked to intra-cellular mRNA localization within the cytoplasm. The intestinal epithelium is a monolayer of epithelial cells that have basal sides that face the blood stream and apical sides that face the lumen. In a recent study, Moor et al. uncovered global apico-basal polarization of mRNAs of around 30% of the genes in intestinal epithelial cells.[28] Strikingly, the localization of mRNAs did not generally match those of the encoded proteins. Rather, ribosomes were more abundant on the apical sides of the cells and consequently apical mRNAs were more efficiently translated (Figure 7A). In response to feeding, mRNAs of specific genes translocated from the ribosome-depleted basal cell side to the ribosome-enriched apical cell side, with an associated specific rapid increase in their translation. β-cells are also highly polarized epithelial cells (Figure 7B), with a basal side facing a vein and apical side surrounded by an arteriole.[54–58] Distinct β-cell faces have been shown to have different properties. The lateral sides are enriched with molecules such as the glucose transporter Glut2[59] and are the sides of more active insulin secretion[60] whereas the apical sides are sites of primary cilia projections.[55] smFISH opens avenues for exploring mRNA polarization and its impact on translational control in the cytoplasm of β-cells in the intact pancreas.

9 | MICRORNA AND RNA BINDING PROTEINS COULD POTENTIALLY RESOLVE THE ABUNDANCE-RESPONSE TRADE-OFF

We have highlighted a fundamental trade-off associated with mRNA lifetime-achieving an elevated steady state mRNA level and minimizing gene expression noise requires an elevated mRNA lifetime. This, however, comes at a cost of slowing down the time required to shift between distinct steady states. A potential regulatory mechanism to alleviate this trade-off is to specifically increase mRNA degradation rates in times of shifts in steady state levels (Figure 2C,D). Alterations of mRNA lifetime can be regulated by RNA binding proteins and microRNAs.[61] MicroRNAs, which are incorporated into the RNA-induced silencing complex (RISC) and accelerate RNA degradation, are attractive regulatory molecules, as their active form can be specifically produced in a rapid manner without the need for protein translation.[62] Indeed, microRNAs carry important roles in maintaining β-cell function and identity.[63,64] It will be interesting to assess the role of microRNA in facilitating rapid shifts in steady states during feeding/fasting, particularly for the highly abundant islet genes. Furthermore, microRNAs and the accumulation of translationally repressed messenger ribonucleoproteins in P-bodies are suggested to play an important role in

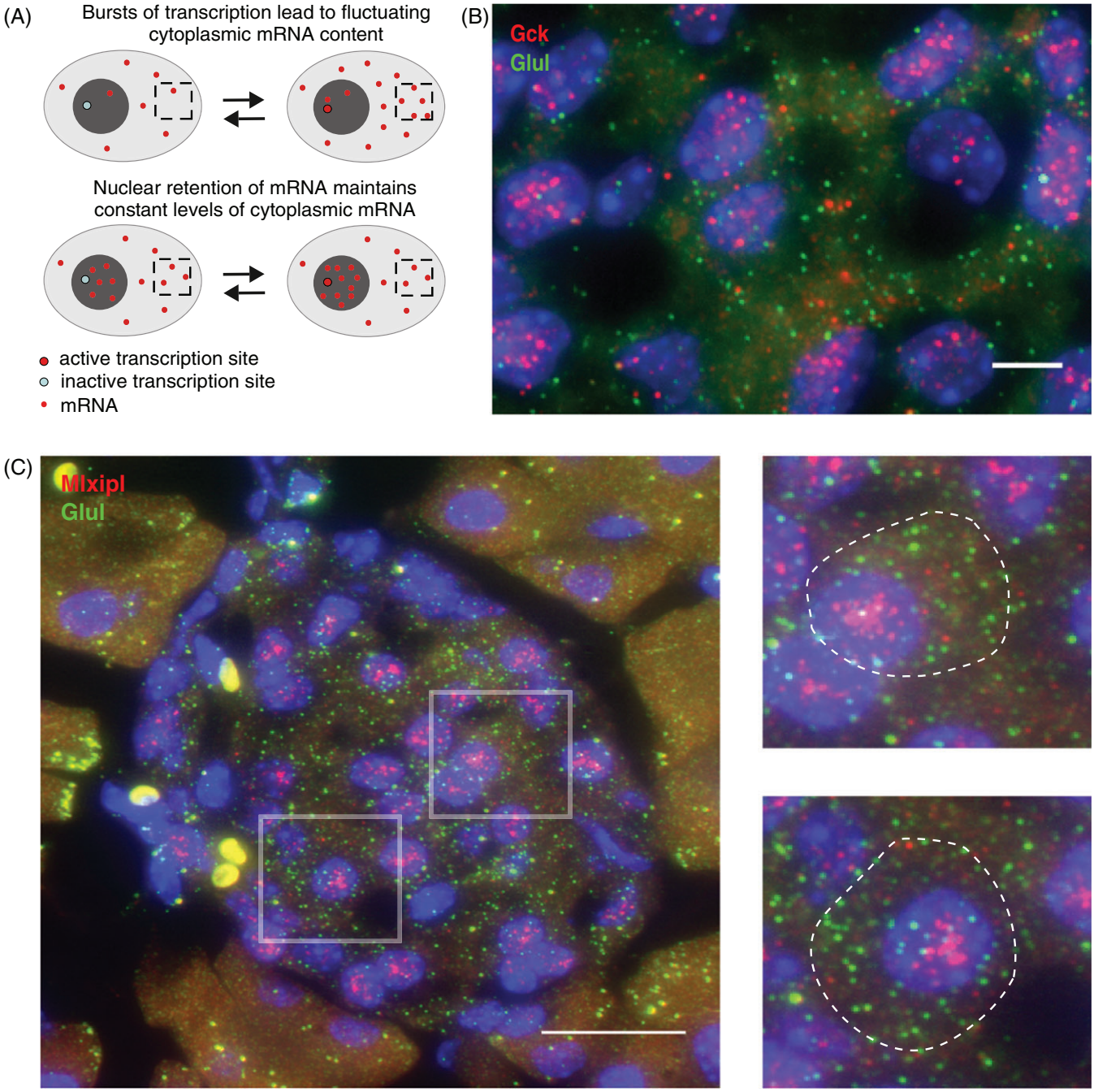

FIGURE 6 Nuclear retention of mRNA can buffer gene expression noise. A, Diagram demonstrating the effect of nuclear retention on cytoplasmic variability in mRNA content. B,C, mRNA molecules of Gck (red, B) and Mlxipl (red, C) are mainly observed in the nucleus of pancreatic β-cells whereas Glul (green) is mainly observed in the cytoplasm. DAPI is in blue. Scale bar is 5 μm (B), 20 μm (C)

inhibiting mRNA translation.[65,66] These features become important for regulating and fine-tuning translation events upon rapid changes and could also enable highly abundant transcripts such as insulin mRNA to rapidly change the steady state of translation.

10 | OUTLOOK

Single molecule approaches are emerging as powerful tools to interrogate the processes of transcription, mRNA degradation, intra-cellular localization and translational control in single cells within intact tissues. The ability to visualize these processes with high sensitivity and spatial resolution offers unique opportunities to characterize the basic processes of gene regulation in the pancreas and in other metabolic organs, and to identify pathological features of miss-regulation in diabetes. A limitation of single molecule FISH is its low-throughput-due to limitations on the numbers of fluorophores that can be simultaneously detected one cannot typically visualize more than 3 to 4 genes in a smFISH experiment. New technologies enable dramatically expanding this limit to hundreds of genes simultaneously detected in the same cell, using combinatorial labelling of probe libraries and multiple rounds of hybridizations.[67–70] Other approaches combine smFISH with massively parallel RNAseq to characterize with higher detail sub-populations of cells with particular transcriptional features.[9,71–74] Combining smFISH

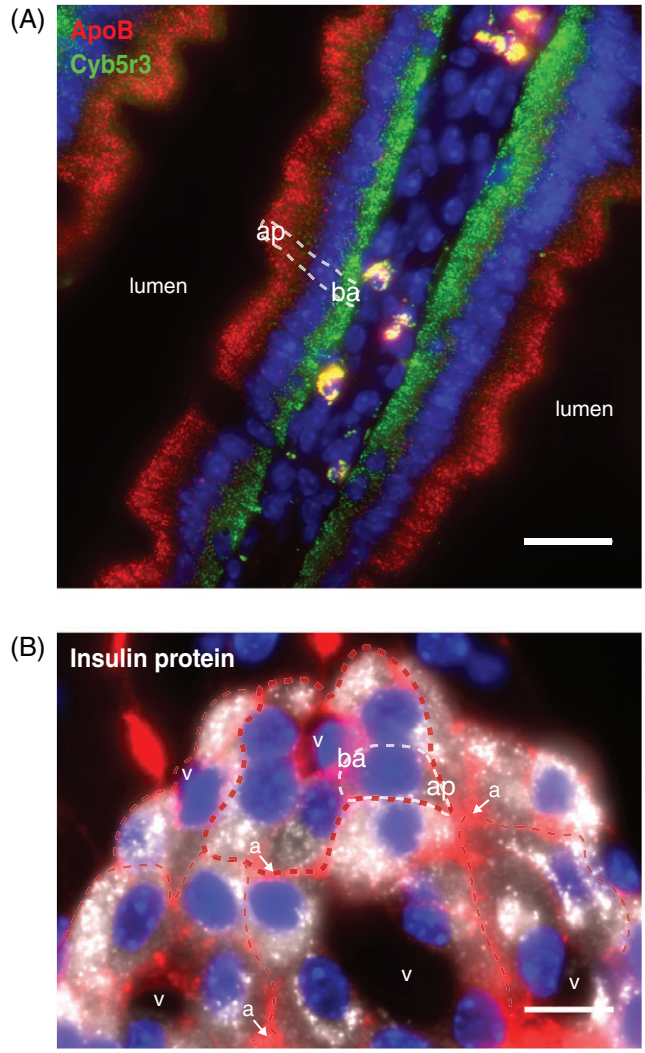

FIGURE 7 Patterns of mRNA polarization along the apico-basal axis in epithelial tissues. A, the intestinal vilus is composed of a monolayer of enterocytes, which absorb nutrients on the apical (ap) side from the lumen and excrete them on the basal side (ba) of the cell into the blood. Moor et al demonstrated that cytoplasmic polarization of mRNA—such as ApoB (red) on the apical side and Cyb5r3 (green) on the basal side—can affect translation due to apical polarization of ribosomes.[28] DAPI is in blue. Scale bar is 20 μm. B, Pancreatic β-cells also exhibit an apico-basal polarization. They assemble in rosette-like structures with a basal side (Ba) facing a vein (v) and an apical side (ap) facing the surrounding arteriole (a). Exploring mRNA polarization under different metabolic and pathological states can shed new light on the process of translational control in the cytoplasm. DAPI is in blue, phalloidin in red marks the cell membrane, and insulin protein in gray

labelling with sorting techniques offers additional avenues for the wider and deeper characterization of such cells.[75–77] These new techniques could expose the design principles that govern gene regulation in β-cells and other cell types in our metabolic organs.

ACKNOWLEDGEMENTS

S.I. is supported by the Henry Chanoch Krenter Institute for Biomedical Imaging and Genomics, The Leir Charitable Foundations, Richard Jakubskind Laboratory of Systems Biology, Cymerman-Jakubskind Prize, The Lord Sieff of Brimpton Memorial Fund, the I- CORE program of the Planning and Budgeting Committee and the Israel Science Foundation (grants 1902/12 and 1796/12), the Israel Science Foundation grant No. 1486/16, the EMBO Young Investigator Program and the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement number 335122, the Bert L. and N. Kuggie Vallee Foundation and the Howard Hughes Medical Institute (HHMI) international research scholar award. S.I. is the incumbent of the Philip Harris and Gerald Ronson Career Development Chair.

Conflict of interest

The authors declare that they have no conflict of interest.

ORCID

Shalev Itzkovitz http://orcid.org/0000-0003-0685-2522

REFERENCES

1. Wasserman DH. Four grams of glucose. *Am J Physiol Endocrinol Metab*. 2009;296:E11–E21.
2. Moor AE, Itzkovitz S. Spatial transcriptomics: paving the way for tissue-level systems biology. *Curr Opin Biotechnol*. 2017;46:126–133.
3. Itzkovitz S, Oudenaarden A. Validating transcripts with probes and imaging technology. *Nat Methods*. 2011;8:S12–S19.
4. Quake, SR, Wyss-Coray, T, Darmanis S. Transcriptomic characterization of 20 organs and tissues from mouse at single cell resolution creates a tabula muris. *bioRxiv* (2017). https://doi.org/10.1101/237446
5. Regev, A, Teichmann SA, Lander ES, et al. Human Cell Atlas Meeting Participants The Human Cell Atlas. *bioRxiv* 121202 (2017). https://doi.org/10.1101/121202, 6
6. Colnot S, Perret C. Liver zonation. In: Monga SPS, ed. *Molecular Pathology of Liver Diseases*. Springer US, Boston, MA; 2011:7–16.
7. Gebhardt R. Metabolic zonation of the liver: regulation and implications for liver function. *Pharmacol Ther*. 1992;53:275–354.
8. Jungermann K, Keitzmann T. Zonation of parenchymal and nonparenchymal metabolism in liver. *Annu Rev Nutr*. 1996;16:179–203.
9. Bahar Halpern K et al. Single-cell spatial reconstruction reveals global division of labour in the mammalian liver. *Nature*. 2017;542:352–356.
10. Gutierrez GD, Gromada J, Sussel L. Heterogeneity of the pancreatic Beta cell. *Front Genet*. 2017;8:22. http://doi.org/10.3389/fgene.2017.00022
11. Dorrell C, Schug J, Canaday PS, et al. Human islets contain four distinct subtypes of β cells. *Nat Commun*. 2016;7:11756.
12. Kiekens R, in 't Veld P, Mahler T, Schuit F, van de Winkel M, Pipeleers D. Differences in glucose recognition by individual rat pancreatic B cells are associated with intercellular differences in glucose-induced biosynthetic activity. *J Clin Invest*. 1992;89:117–125.
13. Migliorini A, Lickert H, Gegg M, Roscioni SS. Impact of islet architecture on β-cell heterogeneity, plasticity and function. *Nat Rev Endocrinol*. 2016;12:695.
14. Salomon D, Meda P. Heterogeneity and contact-dependent regulation of hormone secretion by individual B cells. *Exp Cell Res*. 1986;162:507–520.
15. Van De Winkel M, Pipeleers D. Autofluorescence-activated cell sorting of pancreatic islet cells: purification of insulin-containing B-cells according to glucose-induced changes in cellular redox state. *Biochem Biophys Res Commun*. 1983;114:835–842.
16. Bader E, Migliorini A, Gegg M, et al. Identification of proliferative and mature β-cells in the islets of Langerhans. *Nature*. 2016;535:430–434.

17. Johnston NR, Mitchell RK, Haythorne E, et al. Beta cell hubs dictate pancreatic islet responses to glucose. *Cell Metab*. 2016;24:389–401.
18. Baron M et al. A single-cell transcriptomic map of the human and mouse pancreas reveals inter- and intra-cell population structure. *Cell Syst*. 2016;3:346–360.e4.
19. Li J, Klughammer J, Farlik M, et al. Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. *EMBO Rep*. 2016;17:178–187.
20. Muraro MJ, Dharmadhikari G, Grün D, et al. A single-cell transcriptome atlas of the human pancreas. *Cell Systems*. 2016;3:385–394.e3.
21. Segerstolpe Å, Palasantza A, Eliasson P, et al. Single-cell transcriptome profiling of human pancreatic islets in health and type 2 diabetes. *Cell Metab*. 2016;24:593–607.
22. Wang YJ, Schug J, Won KJ, et al. Single-cell transcriptomics of the human endocrine pancreas. *Diabetes*. 2016;65:3028–3038.
23. Femino AM, Fay FS, Fogarty K, Singer RH. Visualization of single RNA transcripts in situ. *Science*. 1998;280:585–590.
24. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods*. 2008;5:877–879.
25. Itzkovitz S, Lyubimova A, Blat IC, et al. Single molecule transcript counting of stem cell markers in the mouse intestine. *Nat Cell Biol*. 2011;14:106–114.
26. Bahar Halpern K, Tanami S, Landen S, et al. Bursty gene expression in the intact mammalian liver. *Mol Cell*. 2015;58:147–156.
27. Bahar Halpern K, Itzkovitz S. Single molecule approaches for quantifying transcription and degradation rates in intact mammalian tissues. *Methods*. 2016;98:134–142.
28. Moor AE, Golan M, Massasa EE, et al. Global mRNA polarization regulates translation efficiency in the intestinal epithelium. *Science*. 2017;357:1299–1303.
29. Rabani M, Levin JZ, Fan L, et al. Metabolic labeling of RNA uncovers principles of RNA production and degradation dynamics in mammalian cells. *Nat Biotechnol*. 2011;29:436–442.
30. Schwanhäusser B, Busse D, Li N, et al. Global quantification of mammalian gene expression control. *Nature*. 2011;473:337–342.
31. Alon U. *An Introduction to Systems Biology: Design Principles of Biological Circuits*. Chapman and Hall/CRC, Boca Raton , FL; 2006.
32. Golding I, Paulsson J, Zawilski SM, Cox EC. Real-time kinetics of gene activity in individual bacteria. *Cell*. 2005;123:1025–1036.
33. Raj A, Oudenaarden A v. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell*. 2008;135:216–226.
34. Eldar A, Elowitz MB. Functional roles for noise in genetic circuits. *Nature*. 2010;467:167–173.
35. Dar RD, Razooky BS, Singh A, et al. Transcriptional burst frequency and burst size are equally modulated across the human genome. *Proc Natl Acad Sci U S A*. 2012;109:17454–17459.
36. Suter DM, Molina N, Gatfield D, Schneider K, Schibler U, Naef F. Mammalian genes are transcribed with widely different bursting kinetics. *Science*. 2011;332:472–474.
37. Blake WJ, Balázsi G, Kohanski MA, et al. Phenotypic consequences of promoter-mediated transcriptional noise. *Mol Cell*. 2006;24:853–865.
38. Chen X, Chen Z, Chen H, et al. Nucleosomes suppress spontaneous mutations base-specifically in eukaryotes. *Science*. 2012;335:1235–1238.
39. Mueller F, Stasevich TJ, Mazza D, McNally JG. Quantifying transcription factor kinetics: at work or at play? *Crit Rev Biochem Mol Biol*. 2013;48:492–514.
40. Selby CP, Drapkin R, Reinberg D, Sancar A. RNA polymerase II stalled at a thymine dimer: footprint and effect on excision repair. *Nucleic Acids Res*. 1997;25:787–793.
41. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860–921.
42. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science*. 2001;291:1304–1351.
43. Welsh M, Nielsen DA, MacKrell AJ, Steiner DF. Control of insulin gene expression in pancreatic beta-cells and in an insulin-producing cell line, RIN-5F cells. II. Regulation of insulin mRNA stability. *J Biol Chem*. 1985;260:13590–13594.
44. Oeffinger M, Zenklusen D. To the pore and through the pore: a story of mRNA export kinetics. *Biochim Biophys Acta*. 2012;1819:494–506.
45. Shav-Tal Y, Darzacq X, Shenoy SM, et al. Dynamics of single mRNPs in nuclei of living cells. *Science*. 2004;304:1797–1800.
46. Vargas DY, Raj A, Marras SAE, Kramer FR, Tyagi S. Mechanism of mRNA transport in the nucleus. *PNAS*. 2005;102:17008–17013.
47. Bahar Halpern K, Caspi I, Lemze D, et al. Nuclear retention of mRNA in mammalian tissues. *Cell Rep*. 2015;13:2653–2662.
48. Battich N, Stoeger T, Pelkmans L. Control of transcript variability in single mammalian cells. *Cell*. 2015;163:1596–1610.
49. Singh A, Bokes P. Consequences of mRNA transport on stochastic variability in protein levels. *Biophys J*. 2012;103:1087–1096.
50. Xiong L, Ma Y, Tang L. Attenuation of transcriptional bursting in mRNA transport. *Phys Biol*. 2010;7:016005.
51. Spector DL. Nuclear domains. *J Cell Sci*. 2001;114:2891–2893.
52. Prasanth KV, Prasanth SG, Xuan Z, et al. Regulating gene expression through RNA nuclear retention. *Cell*. 2005;123:249–263.
53. Itoh N, Okamoto H. Translational control of proinsulin synthesis by glucose. *Nature*. 1980;283:100–102.
54. Bonner-Weir S. Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans. *Diabetes*. 1988;37:616–621.
55. Gan WJ, Zavortink M, Ludick C, et al. Cell polarity defines three distinct domains in pancreatic β-cells. *J Cell Sci*. 2017;130:143–151.
56. Geron E, Boura-Halfon S, Schejter ED, Shilo B-Z. The edges of pancreatic islet β cells constitute adhesive and signaling microdomains. *Cell Rep*. 2015;10:317–325.
57. Granot Z, Swisa A, Magenheim J, et al. LKB1 regulates pancreatic β cell size, polarity, and function. *Cell Metab*. 2009;10:296–308.
58. Weir GC, Bonner-Weir S. Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. *J Clin Invest*. 1990;85:983–987.
59. Orci L, Thorens B, Ravazzola M, Lodish HF. Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. *Science*. 1989;245:295–297.
60. Takahashi N, Kishimoto T, Nemoto T, Kadowaki T, Kasai H. Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. *Science*. 2002;297:1349–1352.
61. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136:215–233.
62. Giraldez AJ, Mishima Y, Rihel J, et al. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*. 2006;312:75–79.
63. Melkman-Zehavi T, Oren R, Kredo-Russo S, et al. miRNAs control insulin content in pancreatic β-cells via downregulation of transcriptional repressors. *EMBO J*. 2011;30:835–845.
64. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature*. 2004;432:226–230.
65. Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol*. 2005;7:719–723.
66. Poy MN, Spranger M, Stoffel M. microRNAs and the regulation of glucose and lipid metabolism. *Diabetes Obes Metab*. 2007;9:67–73.
67. Lubeck E, Coskun AF, Zhiyentayev T, Ahmad M, Cai L. Single-cell in situ RNA profiling by sequential hybridization. *Nat Methods*. 2014;11:360–361.
68. Moffitt JR, Zhuang X. Chapter one - RNA imaging with multiplexed error-robust fluorescence in situ hybridization (MERFISH). In: Jaffrey GSF, ed. *Methods in Enzymology*. Vol 572. Academic Press; 2016:1–49. https://doi.org/10.1016/bs.mie.2016.03.020
69. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science*. 2015;348:aaa6090.
70. Shah S, Lubeck E, Zhou W, Cai L. In situ transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus. *Neuron*. 2016;92:342–357.
71. Achim K, Pettit JB, Saraiva LR, et al. High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin. *Nat Biotechnol*. 2015;33:503–509.
72. Crosetto N, Bienko M, van Oudenaarden A. Spatially resolved transcriptomics and beyond. *Nat Rev Genet*. 2015;16:57–66.

73. Moor AE, Harnik Y, Ben-Moshe S, Massasa EE, Bahar Halpern, K, Itzkovitz, S. Spatial reconstruction of single enterocytes uncovers broad zonation along the intestinal villus axis. bioRxiv 261529 (2018). https://doi.org/10.1101/261529
74. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. *Nat Biotechnol*. 2015;33:495–502.
75. Klemm S, Semrau S, Wiebrands K, et al. Transcriptionally profiling cells sorted by transcript abundance. *Nat Methods*. 2014;11:549–551.
76. Bushkin Y, Radford F, Pine R, et al. Profiling T cell activation using single molecule-FISH and flow cytometry. *J Immunol*. 2015;194:836–841.
77. Rouhanifard SH, Dunagin M, Mellis IA, et al. Single-molecule fluorescent amplification of RNA using clampFISH probes. bioRxiv 222794 (2017). https://doi.org/10.1101/222794

How to cite this article: Farack L, Egozi A, Itzkovitz S. Single molecule approaches for studying gene regulation in metabolic tissues. *Diabetes Obes Metab*. 2018;20(Suppl. 2):145–156. https://doi.org/10.1111/dom.13390