Early Career Research Award

Metabolic sensors and their interplay with cell signalling and transcription

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Abstract

There is an intimate, yet poorly understood, link between cellular metabolic status, cell signalling and transcription. Central metabolic pathways are under the control of signalling pathways and, vice versa, the cellular metabolic profile influences cell signalling through the incorporation of various metabolic sensors into the signalling networks. Thus information about nutrients availability directly and crucially influences crucial cell decisions. In the present review, I summarize our current knowledge of various metabolic sensors and give some examples of the integration of metabolically derived inputs into the signalling system and the regulation of transcription. I also discuss the Warburg effect where the cross-talk between metabolism and signalling is used to orchestrate rapid cell growth and division. It is becoming clear that future research will concentrate on the collection of small-molecule metabolites, whose concentration fluctuates in response to cellular energy levels, searching for their sensors that connect them to the signalling and transcriptional networks.

Key words: energy status, metabolic sensor, signalling, small metabolite, transcription, Warburg effect.

Abbreviations used: ADPR, ADP-ribose; AMPK, AMP-dependent protein kinase; APC/C, anaphase-promoting complex/cyclosome; ART, ADP-ribosyltransferase; cADPR, cADP-ribose; CBP, CREB (cAMP-response-element-binding protein)-binding protein; CREB, cAMP-response-element-binding protein; CtBP, C-terminal binding protein; FIH1, factor inhibiting hypoxia-inducible factor-1; FoxO, forkhead box O; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; IDH, isocitrate dehydrogenase; JmjC, Jumonji C; LSD1, lysine-specific demethylase 1; MAP4K3, mitogen-activated protein kinase kinase kinase kinase 3; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; NMNAT-1, nicotinamide mononucleotide adenylate transferase-1; OAADPR, O-acetyl-ADP-ribose; PARP, poly(ADP-ribose) polymerase; PGC1α, peroxisome-proliferator-activated receptor γ co-activator 1α; PHD protein, prolyl hydroxylase domain-containing protein; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome-proliferator-activated receptor γ; PRAS40, proline-rich Akt substrate of 40 kDa; raptor, regulatory associated protein of mTOR; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine; TRPM2, transient receptor potential melastatin 2; Vps, vacuolar protein sorting.

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The link between the cellular power station and crucial cell processes

In order to survive, cells need to produce a constant supply of energy and to continuously derive the necessary precursors for the synthesis of proteins, nucleic acids and membranes. The main sources of energy in an animal cell are glucose, the amino acid glutamine and fatty acids, all supplied mainly from the bloodstream. In the cell cytoplasm, sugars are broken down into pyruvate during glycolysis that is then transported to the mitochondrial matrix and converted into acetyl-CoA and processed in the tricarboxylic acid cycle. The mitochondrial matrix is also the location where fatty acids are metabolized during β-oxidation to produce acetyl-CoA which is again processed further in the tricarboxylic acid cycle. Similarly, glutamine feeds into the tricarboxylic acid cycle following its conversion into glutamate. Intermediates of the tricarboxylic acid cycle then serve as precursors for the synthesis of non-essential amino acids and lipids, and, together with the pentose-phosphate pathway, are involved in the production of the precursors for the synthesis of nucleotides. Whereas glucose and fatty acids contribute carbon, oxygen and hydrogen for the anabolic/synthetic processes, glutamine provides the source of nitrogen. There are several thousands of metabolites in the cells that take part in the metabolic processes. Some of them, such as ATP or NAD(P)(H), are shared by several pathways and therefore serve as good indicators of the overall metabolic status of the cell. However, only a few sensors of a handful of metabolites have been identified so far and their interconnection to the signalling networks remains elusive.

Although historically finding itself at the centre of biochemists' attention in the early 20th Century, the boom of molecular biology has, to some extent, put the research

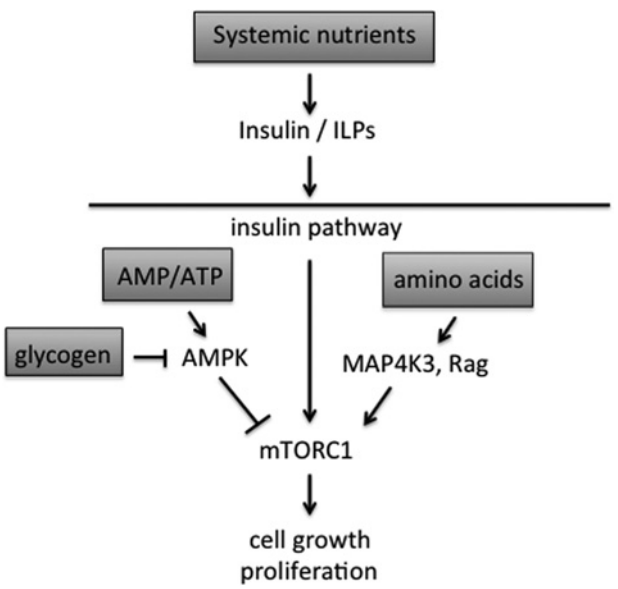
of metabolic pathways out of the spotlight. However metabolic enzymes are under the tight control of various cell signalling pathways and transcription factors. Parallel evidence is emerging that details the crucial roles of various metabolic sensors and feedback mechanisms in sensing and integrating dynamic changes in cellular metabolic states. Moreover, these are able to mediate appropriate responses in crucial cellular processes, such as cell signalling, chromatin structure/function and ultimately gene expression. Therefore it appears that not only is a cell's metabolic status subject to extrinsic controls, but also this status can directly regulate the activity of other crucial cellular pathways that do not necessarily only exist to regulate metabolic activity. In the present review, I discuss the known metabolic sensors and I illustrate with a few elegant examples how cellular metabolism integrates with cell signalling and the regulation of transcription.

Metabolic sensors regulating cell growth and division

Specific pathways have evolved that constantly monitor the levels of systemic nutrients, amino acids and cellular energy. These all converge on a key protein complex known as mTORC [mTOR (mammalian target of rapamycin) complex] 1 (named after one of its components, the rapamycin-sensitive mTOR kinase). mTORC1 is a positive regulator of protein synthesis, cell growth and cell-cycle progression, and is active when nutrients are abundant [1]. In addition to driving anabolism, mTORC1 is also able to mediate the cellular balance between energy storage and consumption. The complex exists as a dimer containing the mTOR kinase, a negative regulator PRAS40 (proline-rich Akt substrate of 40 kDa) and a protein called raptor (regulatory associated protein of mTOR) that functions as a scaffold for assembling the complex and that is also involved in the binding of both substrates and other regulatory proteins [2]. In addition, mTORC1 shares two other proteins [mLST8 (mammalian lethal with sec-13 protein 8) and deptor (DEP domain-containing mTOR-interacting protein)] with the closely related mTORC2. However, it should be noted that mTORC2 is rapamycin-insensitive and has been shown to act functionally upstream of mTORC1. Among the well-characterized downstream targets of mTORC1 are the S6K1 (S6 kinase-1) that promotes the elongation of protein synthesis and a negative regulator of translation initiation called 4E-BP1 (eukaroytic initiation factor 4E-binding protein 1) that dissociates from mRNAs after the phosphorylation by mTORC1 [3]. mTORC1 has also been demonstrated to regulate the transcription of rRNA genes and promote ribosomal biogenesis by regulating the expression of the transcription factor Myc, the association between UBF (upstream binding factor) and SL1 (selectivity factor 1) and by activation of TIF-1A (transcription initiation factor 1A). Thus mTORC1 can be conceptualized as a gatherer of multiple levels information that then directs appropriate cellular responses, thus crucially influences cell decisions, in response to cellular metabolic status. Comprehensive reviews about mTORC1 have been published elsewhere [4–6]. However, I will briefly summarize the main inputs that feed on mTORC1 because they serve as metabolic sensors with an impact on cell signalling (Figure 1).

Figure 1 | Metabolic sensors involved in the regulation of cell growth

Three metabolism-sensing pathways converge on mTORC1 that stimulates cell growth and proliferation under the conditions of abundant nutrients. (i) Cellular energy levels are sensed through AMPK. A high AMP/ATP ratio activates AMPK that inhibits mTORC1, thus preventing cell growth under the conditions of low cellular energy status. Conversely, glycogen inhibits AMPK directly to allow cell growth. (ii) Systemic nutrients (mainly blood glucose and amino acid levels) trigger the release of insulin and insulin-like peptides (ILPs) that activate the insulin pathway stimulating mTORC1 and thus synchronizing individual cell growth with the growth of the rest of the organism. (iii) The level of amino acids is monitored via MAP4K3 upstream of the Rag small GTPases that positively regulate mTORC1.



AMPK (AMP-dependent protein kinase)

Cellular energy levels are monitored via the activation of AMPK. AMPK exists as a heterotrimer of α-, β- and γ-subunits and, as its name suggests, it is activated by a high AMP/ATP ratio when cellular energy levels are either low or declining. AMP binds to the regulatory γ-subunit of the kinase that in turn allows the catalytic α-subunit to become active after its phosphorylation by the upstream LKB1 kinase. Active AMPK blocks cellular growth by inhibiting mTORC1 [7], thus ensuring the preservation of already scarce energy levels. Conversely, AMPK can also be negatively regulated by glycogen that binds to the β-subunit, thus permitting mTORC1-mediated cell growth when cellular energy reserves are high [8]. Besides its role in regulating cell growth, AMPK activation has also been

shown to maintain both planar cell polarity and epithelial integrity in an energy-dependent fashion [9].

Amino acids

The levels of amino acids, as the building blocks for protein synthesis, are also monitored on an individual cell basis. In fact, amino acids are absolutely required for mTORC1 signalling and cannot be overruled for by other mTORC1-activating stimuli [10]. Although the identity and action of the primary amino acid sensor is still unknown, downstream mediators have emerged. The Rag proteins are a group of small GTPases that associate with raptor, a component of mTORC1. They physically interact with and are activated by MAP4K3 (mitogen-activated protein kinase kinase kinase kinase 3) [11], although the mechanism of their action on mTORC1 is not clear. However, recent evidence suggests that amino acid signalling through Rag GTPases causes mTORC1 to shuttle to lysosomal membranes [12]. The significance of this shuttling is not clear, but it may provide a physical link between amino acid sensing and the inhibition of autophagy (i.e. a process of self-digestion of redundant cellular components triggered under an energetic stress which is dependent on the correct function of lysosomes) and/or provide a docking site for the integral activation of mTORC1 from various other inputs. Another protein that has been linked to amino acid sensing is Vps (vacuolar protein sorting) 34, a type III PI3K (phosphoinositide 3-kinase) [13]. However, the strength of this relationship to any of the above discussed components still requires more thorough investigation at this stage.

Insulin pathway

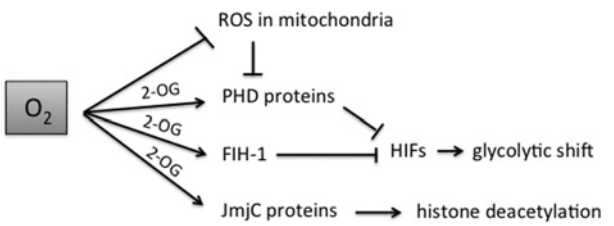
The co-ordination between the individual cell growth and the overall growth of the organism is mediated through the insulin pathway. This importance was first noted in *Drosophila*, in which mutants of several components of the insulin pathway showed severely reduced body size [14]. Following feeding, insulin and insulin-like peptides circulate in the blood at high levels where they bind to the insulin receptor tyrosine kinases and influence cell growth via the conserved PI3K/Akt [also known as PKB (protein kinase B)] signalling pathway. The catalytic subunit of PI3K phosphorylates PtdIns(4,5)P_2 in the plasma membrane, converting it into PtdInsP_3. PtdInsP_3 then recruits two subsequent protein kinases, PDK1 (phosphoinositide-dependent kinase 1) and Akt, leading to the activation of Akt that then regulates several growth modulators, mainly mTORC1 through the inhibition of TSC2 (tuberous sclerosis complex 2) and PRAS40 [15].

Oxygen sensors: the connection between oxygen, 2-oxoglutarate, iron and ascorbic acid

Most cells are able to respond to oxygen levels using three interconnected sensors (Figure 2): (i) PHD proteins (prolyl hydroxylase domain-containing proteins), (ii) FIH1 [factor inhibiting HIF (hypoxia-inducible factor)-1], and (iii) mitochondria. Together, these sensors regulate the activity of HIFs that in turn orchestrate transcriptional responses that mediate cell adaptation under hypoxic conditions, including the switch from oxidative to glycolytic metabolism [16,17]. However, it should be noted that recent findings suggest that histone demethylases containing the so-called JmjC (Jumonji C) domain also function as oxygen sensors and, as a consequence, chromatin acts as an oxygen-responsive structure [18].

Figure 2 | Cellular sensors for oxygen

Both the PHD proteins (i) and FIH1 (ii) require oxygen to inhibit the action of HIFs under the normoxic conditions. Low oxygen levels trigger the production of ROS in mitochondria (iii) that inactivate the PHD proteins leading to the activation of HIFs. Histone demethylases (iv) with the JmjC domain also require oxygen for their enzymatic activity. Moreover, during their catalytic reactions, the PHD proteins, FIH1 and JmjC convert 2-oxoglutarate (2-OG) into succinate and CO_2, thus linking oxygen sensing with the tricarboxylic acid cycle and possibly amino acid sensing. Iron and ascorbic acid are required as cofactors.



HIFs exist as heterodimers comprising an oxygen-sensitive HIFα subunit and an oxygen-insensitive HIFβ subunit. Under normoxia (conditions of normal oxygen concentration), HIFα is hydroxylated by the action of PHD proteins. Such hydroxylation increases HIFα's binding affinity for vHL (von Hippel–Lindau protein), a component of the E3 ubiquitin ligase complex, thus promoting its degradation by the proteasome [19]. Crucially, successful hydroxylation of HIF-1α by PHD proteins not only requires oxygen as a substrate, but also 2-oxoglutarate (and iron and ascorbic acid as cofactors), thus linking oxygen sensing with the activity of the tricarboxylic acid cycle [20,21]. A similar mechanism operates for the asparagyl hydroxylase FIH1, as it is also oxygen- and 2-oxoglutarate-dependent. FIH1 also hydroxylates HIF-1α under conditions of normoxia. However, rather than targeting HIF-1α for degradation, FIH1-mediated hydroxylation prevents HIF-1α's association with the transcriptional co-activators CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300. Such a lack of association prevents HIF-1 bound to chromatin at its DNA-recognition sites from activating target gene transcription and thereby blocks inappropriate expression [22]. However, under hypoxic (oxygen-limiting) conditions, the activity of PHD proteins and FIH1 becomes compromised, owing to their requirement for oxygen as a substrate, and functionally active HIF-1α proteins are stabilized and direct the transcription of genes required to

direct the appropriate cellular response to this type of stress condition; for example, the up-regulation of several mRNAs for enzymes in the glycolytic pathway to mediate the switch from oxidative to anaerobic respiration [16,17,23].

The mitochondrial response to oxygen deprivation is to produce ROS (reactive oxygen species) [24]. Utilizing an as yet unidentified mechanism, ROS are able to inhibit the activity of PHD proteins and thus increase the stability of HIF-1α protein. Interestingly, this effect is not caused by a simple and indirect signalling of mitochondria to PHD proteins via their consumption of oxygen (at the terminal phase of electron transport during oxidative phosphorylation). This is because experimental manipulation of mitochondria in a manner that increases ROS production but does not alter oxygen consumption still results in stabilized HIF-1α [25].

Histone demethylases containing a JmjC domain [JmjC and JHDM (JmjC domain-containing histone demethylase) protein families] also require oxygen and 2-oxoglutarate for their enzymatic function (the removal of post-translationally added methyl groups from lysine and arginine residues of histones and other proteins) [26]. However, to date, only a few studies have addressed the role of these enzymes in oxygen sensing and hypoxia. In hypoxic human bronchial cells, the activity of the JARID1A (Jumonji/AT-rich interactive domain 1A) histone demethylase has been shown to be inhibited, leading to increased levels of the transcriptionally activating associated histone H3 Lys^4 trimethylation (H3K4me3) epigenetic mark in chromatin [27]. Conversely, there is evidence that many of the JmjC family proteins are induced by hypoxia, although how their enzymatic activity is maintained during conditions of low oxygen is yet to be determined [28]. Irrespective of these two apparently contrasting results, a potentially novel role for oxygen sensing by JmjC domain-containing proteins linked to chromatin remodelling and epigenetic control of gene expression remains enticing.

FIH1, PHD and JmjC proteins all belong to the family of Fe(II)- and 2-oxoglutarate-dependent dioxygenase enzymes. During their catalytic reactions, they convert 2-oxoglutarate into succinate and CO_2. Considering that 2-oxoglutarate represents an indicator of amino acid catabolism (mainly as a product of the catabolism of glutamine, one of the main extracellular sources of energy for the cell), the above-mentioned dioxygenases can not only be considered as oxygen sensors, but also be theoretically classified as amino acid sensors [29]. However, their connection to other more characterized amino acid sensors, such as the Rag/Vps3/mTOR pathway, has yet to be described.

Interestingly, all of the 2-oxoglutarate-dependent dioxygenase enzymes described in the present review are inhibited by 2-hydroxyglutarate, as well as the tricarboxylic acid cycle intermediates fumarate and succinate [30]. Some cancer cells use this mechanism to down-regulate 2-oxoglutarate-dependent dioxygenases. For example, glioblastoma cancers have been shown to accumulate 2-hydroxglutarate. This is due to specific point mutations in the genes for IDH (isocitrate dehydrogenase) (IDH1 and IDH2) that furnish the enzyme with a new catalytic activity converting 2-oxoglutarate into 2-hydroxyglutarate [31]. Accumulation of this 'oncometabolite' is thought to cause the activation of the hypoxia-response pathway (via HIF-1α) and up-regulation of the glycolytic enzymes necessary for continued cancer cell growth. Similarly, certain renal cancer cells harbour mutations in the fumarate hydratase gene that lead to the build-up of fumarate, another inhibitor of 2-oxoglutarate-dependent dioxygenase oxygen sensors [32].

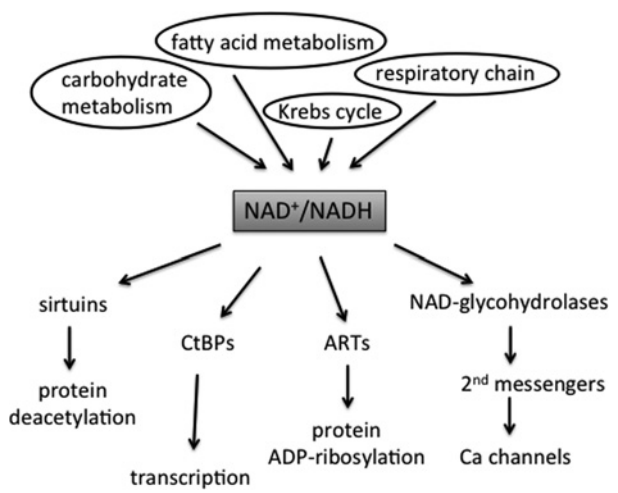
Sensors of NAD^+/NADH and its metabolites

NAD^+ and its reduced form (NADH) play central roles as electron carriers in the oxidoreductase reactions during glycolysis, the tricarboxylic acid cycle, the respiratory chain and other metabolic pathways (Figure 3). Here, NAD^+ serves as a coenzyme that is reversibly converted into NADH or vice versa. In contrast, the role of NAD^+ in other processes involves its irreversible cleavage. Such processes can result in protein deacetylation (sirtuins), mono- or poly-ADP-ribosylation (ADP-ribosyltransferases) or the generation of a whole range of NAD-derived second messengers involved in calcium signalling (NAD-glycohydrolases and also by-products of sirtuins and ADP-ribosylases) [33]. As a consequence, the NAD(H)-consuming molecules can serve as metabolic sensors, but they can also indirectly affect NAD(H) bioavailability, thus having major effects on energy metabolism, cell survival and aging [34].

Owing to the central role played by NAD^+ as a cofactor in classical metabolic pathways, the ratio of NAD^+ to NADH [i.e. the NAD(H)-specific redox potential] can be considered as a readout of the metabolic state of a cell. Indeed, it is this ratio to which NAD/NADH-sensitive proteins respond. For example, as the NAD^+/NADH ratio decreases during muscle cell differentiation, there is an accompanying decrease in the activity of Sir2 protein (an NAD^+-dependent deacetylase enzyme) [35]. However, it is important to note that most of the NAD^+/NADH in the cell exists bound to proteins located within different cellular compartments. Therefore it is difficult to measure, or even estimate, the concentrations of truly free cellular NAD^+ or NADH pools by simple measurements of whole-cell lysates. These limitations can be overcome, to an extent, by deriving the NAD^+/NADH ratio from the concentrations of oxidized and reduced reactants of suitable near-equilibrium reactions within a given subcellular compartment. For example lactate/pyruvate measurements have been used to estimate the NAD^+/NADH ratio in the cytosol [36] and acetoacetate/β-hydroxybutyrate for mitochondria. On the basis of these methods, the NAD^+/NADH ratio in the cytosol of mammalian COS-7 cells was estimated to be 640 [36] and 100–320 in yeast [37]. It is noteworthy that such ratios are 10-fold higher than ratios based on measuring total NAD^+/NADH measurements in whole-cell lysates. It

Figure 3 | **Cellular sensors for NAD$^+$/NADH**

The pool of nicotinamide dinucleotides is shared by the oxidoreductases in the major metabolic pathways and therefore it can be considered as a good indicator of the overall metabolic status of a cell. Several protein sensors evolved whose activity is dependent on the NAD$^+$/NADH ratio to synchronize the cell signalling and transcriptional events with the cellular metabolic state. (i) The family of sirtuins remove the acetyl group from a whole list of other proteins by transferring it on to an NAD$^+$ cofactor. (ii) The recruitment of the CtBP transcriptional cofactors to chromatin can differ depending on its differential NAD$^+$ or NADH binding. (iii) ARTs mediate NAD$^+$-dependent mono- or poly-ADP-ribosylation of target proteins. (iv) NAD-glycohydrolases (as well as sirtuins) produce NAD-derived metabolites activating calcium channels.



is presumed that, owing to free diffusion between the cytosol and the nucleus, that the concentrations of free NAD$^+$ and NADH are the same between these two compartments. In the following subsections, I use the examples of proteins known to interact with NAD(H) and are able to sense changes in the NAD$^+$/NADH ratio and in turn effect this information into functionally important changes for the cell.

CtBP (C-terminal binding protein)

The CtBPs (CtBP1 and CtBP2 in vertebrates) are best known as transcriptional cofactors that operate in the nucleus. However, CtBPs also have cytosolic functions in mediating the fission of Golgi vesicles and in centrosome assembly and have been shown to be an integral part of the neuronal synapse [38]. In the nucleus, CtBP associates with several classes of transcription factors to mediate transcriptional repression via the recruitment of HDACs (histone deacetylases) (HDAC1 and HDAC2), histone lysine methyltransferases [G9a and GLP (G9a-like protein)], LSD1 (lysine-specific demethylase 1) and SUMO (small ubiquitin-related modifier) E3 ligases [hPC2 (human polycomb protein 2) and PIAS1 (protein inhibitor of activated signal transducer and activator of transcription 1)] [39] that remodel target gene chromatin into transcriptionally inactive facultative heterochromatin. Curiously, however, CtBP can also work as a transcriptional activator. In *Drosophila,* CtBP has been shown to activate the transcription of several Wnt target genes. However, this activating effect is only observed during the pulse of Wnt signalling as CtBP returns to its normal co-repressor function in the absence of Wnt signalling [40]. It has been shown that Pygopus, the cofactor in the Wnt transcriptional complex, recruits monomeric CtBP to mediate maximal transcriptional activation during active Wnt signalling, whereas dimeric CtBP is recruited to other Wnt-responsive targets to mediate transcriptional repression [41].

Strikingly, the recruitment of CtBPs to chromatin-associated transcriptional complexes, and hence its potential to effect transcriptional regulation, can be modulated by its ability to bind either NAD$^+$ or NADH as a cofactor. Moreover, this effect appears to be context-dependent. For example, whereas NADH binding stimulates the recruitment of CtBP to *CDH1* (E-cadherin) or *BRCA1* (breast cancer early-onset 1) promoters [42], it inhibits the interactions between CtBP2 and the repressively acting transcription factor NRSF (neuron-restrictive silencing factor)/REST (repressor element 1-silencing transcription factor) [43]. The NAD$^+$/NADH ratio [or, expressed another way, the availability of NAD(H) for CtBP binding] is therefore a critical regulator of CtBP function. However, it has been shown in other contexts that CtBP–transcription factor interactions can also be independent of the NAD$^+$/NADH ratio, as shown in experiments using a CtBP1 mutant defective in NAD$^+$/NADH binding [44].

Although CtBPs show sequence and structural homology with D2-HDHs (D2-hydroxyacid dehydrogenases), which convert lactate into pyruvate in the presence of NADH, its enzymatic activity towards pyruvate is very low. Instead, an intermediate in the methionine salvage pathway, 2-oxo-4-methylthiobutyrate, seems to be a much better substrate for CtBP1 [45], possibly linking CtBP activity with methionine and spermine metabolism.

Sirtuins

There are seven mammalian sirtuins (Sirt1–Sirt7). Sirt1, Sirt2, Sirt6 and Sirt7 are found in the nucleus, Sirt1 and Sirt2 are found in the cytoplasm, and Sirt3 Sirt4 and Sirt5 are found in the mitochondria. They are mostly lysine-specific NAD$^+$-dependent protein deacetylases that catalyse the removal of acetyl groups from histones H3, H4 and H1 (on residues H4K16, H3K9, H3K14 and H1K26) [46], as well as from a myriad of other proteins. The enzymatic mechanism involves the transfer of the acetyl group to be removed on to an NAD$^+$ cofactor and the subsequent release of the deacetylated protein, NAD-derived nicotinamide and OAADPR (O-acetyl-ADP-ribose). Sirt1, Sirt2 and Sirt3 are also able to remove propionylated residues from histone H3 (H3K23), although this has only been demonstrated *in vitro* [47], and Sirt4 and Sirt6 can also function as mono-ADP-ribosyltransferases. Sirtuins are thought to have roles in many cellular functions, and there are numerous studies describing the action of sirtuins in energy metabolism, cellular senescence, DNA repair, circadian rhythm, inflammation,

prion-mediated neurodegeneration, cellular stress and cancer [48–51].

Sirtuins can directly influence the activity of proteins involved in energy metabolism or, alternatively, they can synergize with other metabolism-sensing pathways to orchestrate complex cellular responses [48]. For example, during fasting, Sirt1 works in the liver to deacetylate and therefore activate the transcription factors FoxO (forkhead box O) and PGC1α [PPARγ (peroxisome-proliferator-activated receptor γ) co-activator 1α] and simultaneously inhibit the activity of SREBP (sterol-regulatory-element-binding protein). The combined response to these Sirt1-mediated modifications is to increase the rate of oxidation of fatty acids and gluconeogenesis, while inhibiting lipogenesis [52]. In addition to its functions in stimulating insulin secretion in the pancreas and decreasing fat storage in the adipose tissue, Sirt1 functions as a key mediator of the central nervous system's response to low nutritional availability in the neuroendocrine cells of the hypothalamus [53]. Sirt1 is also involved in the deacetylation and inactivation of HIF-1α under normoxic conditions or those exhibiting a low rate of glycolysis [54]. However, in hypoxia, this inhibitive interaction on HIF-1α is lost and, instead, Sirt1 deacetylates HIF-2α, leading to its activation [55]. At the same time, sirtuins participate in feedback loops where their own expression is regulated in response to the cellular metabolic status by HIF-1 [55], FoxO3a [56] or PPARγ [57].

Sirtuins represent ideal sensors linking protein acetylation levels to cellular metabolic status. This is because all sirtuins require NAD^+ for their function and as a consequence their activity is lower when cells have an ample availability of glucose and their glycolytic rate is high, thus reducing and maintaining a low NAD^+/NADH ratio. However, as glucose availability decreases causing a comparative lack of cellular energy, the associated increases in the NAD^+/NADH ratio allow sirtuins to become more active [58]. Recent studies have revealed that virtually every enzyme in the glycolytic and gluconeogenic pathways, the tricarboxylic acid and urea cycles as well as fatty acid and glycogen metabolic pathways have been found to be acetylated in human liver tissue [59]. It was found that acetylation positively influenced enzymes that are involved in glycolysis, tricarboxylic acid cycle and fatty acid oxidation, whereas it had a negative effect on the enzymes of the gluconeogenic pathway. Given this evidence and the fact that sirtuins are more active during nutrient-limiting conditions, an attractive hypothesis has been proposed by Leonard Guarente [60] that states that sirtuins could participate in the switch between cell energy storage and energy utilization by direct deacetylation of metabolic enzymes. Despite this concept not yet being proved by showing a direct deacetylation of metabolic enzymes by sirtuins, it remains an extremely exciting possibility.

Interestingly, an enzyme involved in the salvage pathway of the synthesis of NAD^+, NMNAT-1 (nicotinamide mononucleotide adenylate transferase-1), is found in the nucleus where it is recruited by Sirt1 to target gene promoters. Here NMNAT-1 supplies localized levels of NAD^+ needed for Sirt1-dependent deacetylation of histone H4 at Lys^{16} (H4K16) [61]. In this way, Sirt1 recruitment of NMNAT-1 resembles other examples of chromatin-localized metabolite synthesis, such as of the production of dNTPs at the sites of DNA damage by Tip60-recruited ribonucleotide reductase [62] or S-adenosylmethyltransferase recruitment by MafK to supply fresh substrates for histone methyltransferases [63].

ARTs (ADP-ribosyltransferases)

ARTs are enzymes capable of specific NAD^+-dependent post-translational protein modifications called mono- or poly-ADP-ribosylation. These modifications are transient and can be removed from target proteins by the action of ADP-ribosylhydrolases and poly-ADP-ribosyl-glycohydrolases respectively [64]. The best studied mono-ADP-ribosylation reactions are catalysed by bacterial toxins that, after invading eukaryotic cells, target cellular proteins, for example heterotrimeric G-proteins, and cytoskeletal and mitochondrial proteins [65]. However, mono-ADP-ribosylation of a whole host of proteins is also possible under normal physiological conditions, for example by PARP [poly(ADP-ribose) polymerase)]-12 or PARP-10 [66] or by Sirt6 and Sirt7 [67]. Transcription factors and their co-factors are often mono-ADP-ribosylated, and, interestingly, CtBP falls into this list [68].

PARP-1 is responsible for poly-ADP-ribosylation of histones and other chromatin-modifying proteins and transcription factors. This includes PARP-1 itself when it is recruited to sites of DNA damage. However, PARP-1's catalytic activity is only triggered after binding to DNA/chromatin. The addition of poly-ADP-ribose chains by PARP-1 adds a highly negative charge to the substrate protein that, in turn, promotes its repulsion from the highly negatively charged DNA, thus enabling a more permissive chromatin structure for remodelling and/or DNA repair. Under circumstances when DNA is only moderately damaged, PARP-1 participates in DNA repair processes and promotes cell survival. However, extensive PARP-1 activation, as in the case of large-scale DNA damage, induces depletion of cellular NAD^+ and ATP levels, eventually culminating in cell death [69].

NAD-glycohydrolases

These enzymes catalyse the intramolecular cyclization or hydrolysis of the high-energy bond in NAD^+, between the nicotinamide and ribose moieties, thus generating ADPR (ADP-ribose) or cADPR (cADP-ribose) and nicotinamide. There are only two known mammalian NAD-glycohydrolases, CD38 and CD157, both bound to the extracellular cell surface of immune cells via a GPI (glycosylphosphatidylinositol) anchor [70]. It is thought that here they may be involved in NAD^+ hydrolysis following its release through the ruptured plasma membranes of dying or injured cells [71]. However, and somewhat surprisingly, cADPR can also be detected in the cytoplasm, where it is able to activate ryanodine receptors in the endoplasmatic reticulum and the TRPM2 (transient receptor potential

melastatin 2) calcium channel in the plasma membrane resulting in increased cytoplasmic calcium levels. ADPR and OAADPR (a by-product of protein deacetylation by sirtuins) can also activate TRPM2 channels and promote calcium-mediated intracellular signalling [72].

A specific protein domain in several chromatin-related proteins called the macrodomain is capable of binding NAD-derived metabolites, such as monomeric ADP-ribosyl residues or OAADPR [73]. To date, it has been found in ten human proteins, including the histone variant macroH2A, PARP family members, the chromatin-remodelling enzyme CHD1 (chromodomain helicase DNA-binding protein 1) and DNA repair- and apoptosis-related proteins [74]. In the case of macroH2A, experimental evidence suggests that nucleosomes containing macroH2A adopt a more rigid conformation of chromatin structure that is more resistant to the binding of transcription factors and chromatin-remodelling machinery and thus blocks RNA polymerase II-mediated transcription [75]. It has been postulated that because macroH2A interacts with the by-products of Sirt2-mediated deacetylation reactions (i.e. OAADPR); this could partly explain the mechanism of Sirt2-mediated chromatin silencing [76]. An alternative perspective proffers that macroH2A could simply buffer localized levels of Sirt2-produced OAADPR, thereby preventing allosteric inhibition of Sirt2 by its product. It has also been shown that macroH2A is able to interact with PARP-1 to inhibit its function, although the nature of these interactions might be indirect [77].

Sensors for extracellular nucleotides and adenosine: the purinergic receptors

In addition to their important cellular functions in metabolism, nucleotides and their metabolites serve as extracellular signalling molecules that signal through the purinergic family of membrane receptors. These include the G-protein-coupled receptors for adenosine (P1 receptors) and nucleotides (P2Y receptors for ATP, ADP, UTP, UDP and UDP-glucose) as well as the ligand-gated ion channels for ATP (P2X receptors). Purinergic signalling is involved in a wide variety of short-term physiological processes, including exocrine and endocrine secretion, immune response, inflammation, nociceptive mechanosensory transduction, platelet aggregation and vasodilatation. However, purinergic signalling can also affect more long-term processes exemplified by cell proliferation, differentiation, migration and death during embryonic development [78].

How are nucleotides released outside the cell? Stressed, damaged or dying cells have problems with the integrity of their membrane leading to the leakage of nucleotides, NAD^+ and other metabolites that activate purinergic receptors and NAD-dependent ectoenzymes on the surface of immune cells where they elicit appropriate immune responses [79,80]. However, nucleotide release can also be regulated. For example, ATP and NAD^+ are released as co-neurotransmitters in both the sympathetic and parasympathetic neurons of the peripheral and central nervous systems to regulate a variety of cellular responses, including smooth muscle contraction, thrombus formation and inflammation [81]. Additionally, many other cell types release ATP via the exocytotic pathway or through specific transporters in response to mechanical distortion or hypoxia [82]. Following release, ATP is usually quickly degraded by extracellular pyrophosphatases and 5'-nucleosidases. Such degradation generates adenosine that is then available to activate the P1 class of purinergic receptors [83]. Hence the bioavailability nucleotides and their central participation in many cellular pathways also influences cell–cell signalling in a variety of contexts.

RNA-based sensors: the riboswitches

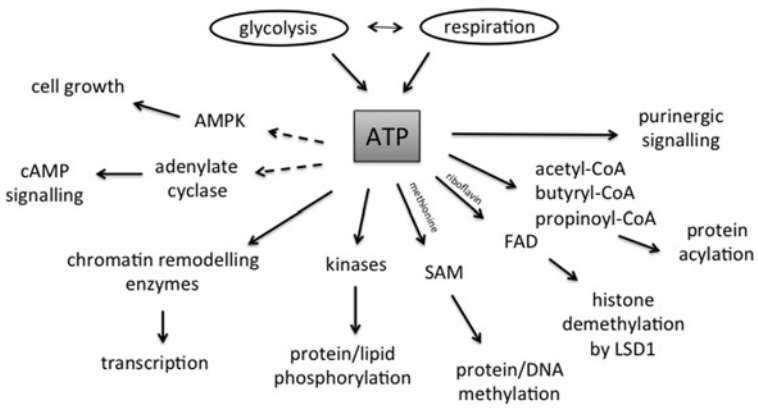
Another elegant way to directly couple metabolism to the regulation of gene expression, splicing and translation is represented by the riboswitches. Instead of protein sensors binding certain metabolites to influence transcription, here metabolites bind directly to the RNA directly itself and effect a 'switch' in its functionality, hence the name riboswitch. Consequently, riboswitches are able to sense a variety of cellular metabolites such as amino acids and their derivatives, carbohydrates, coenzymes, nucleotides and their derivatives, metal ions and tRNAs. Additionally, there are also classes of riboswitch that can respond to changes in temperature and pH [84]. Riboswitch molecules contain ligand-binding aptamer domains that are responsible for causing conformational changes in the coding region of the mRNA that either permit or inhibit translation, only after the binding of an appropriate ligand. In the majority of cases, riboswitches negatively regulate the synthesis or transport of the ligand that they sense. To date, most riboswitches have been described in bacteria, but riboswitches sensing TPP (thiamin pyrophosphate) have been identified in filamentous fungi, green algae and higher plants, where they effect regulation of alternative mRNA splicing [85]. It is noteworthy that naturally occurring riboswitches have also been engineered to function as experimentally useful reporters of gene expression or as biosensors [86].

The link between cellular energy status and protein and DNA modifications

Many proteins involved in cell signalling or regulation of gene expression are subjected to post-translational modifications. The precursors needed for these modifications are often metabolites and their availability is dependent on the metabolic status of a cell. Thus the extent of protein post-translational modifications is, to a large extent, influenced by cellular metabolism. For example, NAD(H) plays a role in protein ADP-ribosylation and deacetylation, as discussed above (Figure 3). ATP has a crucial role in protein phosphorylation, provides energy for functioning of protein motors or chromatin-remodelling enzymes and cAMP plays a signalling role as a second messenger. Biosynthesis of the donors of methyl, acetyl, propionyl or butyryl groups also

Figure 4 | Central role of ATP in the cell signalling and transcriptional events

(i) AMP/ATP levels are sensed by AMPK regulating cell growth. (ii) cAMP is produced by adenylate cyclases from AMP. (iii) Several chromatin-remodelling enzymes require ATP for their action on chromatin. (iv) All kinases involved in the signalling events use ATP as a donor of the phosphate group. (v) SAM is produced from methionine in an ATP-dependent reaction to serve as a substrate for protein and DNA methylation. (vi) FAD is produced from riboflavin in an ATP-dependent reaction (as well as during the oxidation of fatty acids) to serve as a cofactor for the histone demethylase LSD1. (vii) Acetyl-CoA, butyryl-CoA and propionyl-CoA are precursors for post-translational protein acylation. (viii) ATP is released as a co-neurotransmitter on purinergic synapses as well as from stressed, damaged, hypoxic or dying cells to signal through the purinergic receptors.



requires ATP whose availability can influence the levels of the respective protein modifications (Figure 4). In addition, specific lipid or sugar residues modify protein function or cellular localization and signalling properties. I will describe in more detail methylation and acetylation, as well as two recently identified protein modifications: propionylation and butyrylation.

The methylation of DNA, histones and lipids is catalysed by methyltransferases and is dependent on SAM (*S*-adenosylmethionine) serving as a coenzyme. Recently, S-adenosylmethyltransferases have been found not only in the cytoplasm, but also in the nucleus [63]. In the nucleus, S-adenosylmethyltransferases control the localized biosynthesis of SAM that in turn permits the repression of proximal genes by providing histone methyltransferases with the required metabolites to remodel chromatin into transcriptionally repressed structures. Interestingly, they do not perform the same role for DNA methyltransferases [63]. Similarly, the histone demethylase LSD1 requires a cofactor, in this case FAD, in order to remodel chromatin into a transcriptionally repressive state. The synthesis of both SAM and FAD requires ATP, thus providing a link between methylation and cellular energy status [87]. Indeed, it has been shown that high cellular insulin or glucose exposure increases both intracellular SAM concentration and global DNA methylation levels [88]. As mentioned above, the JmjC group of protein demethylases require oxygen and 2-oxoglutarate for their function, thus providing an additional link between methylation levels and cell metabolism. One should note that SAM is also involved in synthesis of polyamines (such as spermidine, spermine and putrescine) which have a highly positive charge and have been shown to bind DNA and to control gene expression at the transcriptional, post-transcriptional and translational levels. Moreover, they also regulate several ion channels [89].

Cytoplasmic acetyl-CoA represents the donor of acetyl groups for protein acetylation reactions. Acetyl-CoA can be synthesized from acetate by the enzyme acetyl-CoA synthetase in yeast as well as in mammals [90]. However, the main source of cytoplasmic acetyl-CoA in mammalian cells derives from citrate by the action of the ATP-dependent enzyme ATP-citrate lyase. The citrate itself is derived from glycolytic precursors (transported into the cytoplasm from the mitochondria), therefore glucose availability can affect global levels of protein acetylation. This includes the acetylation of histones and transcription factors, as well as nearly all of the cytoplasmic enzymes involved in glycolysis, gluconeogenesis, the tricarboxylic acid and urea cycles, fatty acid metabolism and glycogen metabolism [59,91]. The acetylation status of such metabolic enzymes, as discussed in above sections, may be controlled via the NAD^+-dependent deacetylases (class III HDACs and sirtuins) to regulate the switch between glycolytic and oxidative metabolism [60]. Although not experimentally verified, it seems likely that cells utilizing glutamate as their main energy source will be similarly affected by glutamate availability and its shunt into the tricarboxylic acid cycle.

Propionyl-CoA and butyryl-CoA, like acetyl-CoA, are both high-energy products of fatty acid metabolism and are produced through similar chemical reactions [92]. Propionylation and butyrylation have been described on several lysine residues in histones H3, H4 and H2B, as well as in other non-histone proteins [93]. Such modifications are mediated by the same enzymes that are responsible for

protein acetylation, namely p300, CBP and GCN5 (general control non-derepressible 5), but with much lower efficiency than the corresponding acetylation reactions. Propionyl and butyryl groups can also be removed by the action of sirtuins [47]. The function of such modifications remain elusive, although it has been reported that propionylation levels are dynamic during cell differentiation [47].

Regulation of metabolic enzymes by signalling pathways

Considering the extent to which cell signalling is influenced by cellular metabolism, it is perhaps unsurprising that, reciprocally, the enzymes involved in central metabolism are under a control of various signalling pathways. Many of the genes involved in glycolysis are up-regulated by c-Myc [94] and HIF-1α [95]. The PI3K/Akt pathway stimulates glycolysis by up-regulating the expression of glucose transporters and by activation of hexokinase-2 and phosphofructokinase-2. Additionally, active PI3K/Akt signalling stimulates the synthesis of fatty acids [96]. Another player is the transcription factor Oct1 that is involved in the up-regulation of glycolysis and suppression of oxidative respiration [97]. Conversely, p53 works in the opposite way to promote oxidative phosphorylation at the expense of glycolysis [98]. It is important to note that these key regulators, together with a network of other transcriptional factors, such as NF-κB (nuclear factor κB), sirtuins, FoxO, PGC1α, CREB or PPARγ, represent the key convergence points of many different and competing signalling pathways working together to co-ordinate the metabolic state of cells and whole tissues [99–102].

The same factors and metabolic pathways can be utilized by both rapidly dividing cells and cancer cells to satisfy the high energetic demands associated with enhanced growth and replication [103]. Such cells switch from oxidative respiration to anaerobic glycolytic-based metabolism. According to this switch, they also increase their rate of glycolysis. Both the switch to and up-regulation of glycolysis occur, even under conditions of normal oxygen levels that would ordinarily favour oxidative phosphorylation. This phenomenon is known as the Warburg effect after its discoverer [104]. In cells exhibiting the Warburg effect, pyruvate is not transported to mitochondria and is therefore not converted into acetyl-CoA and does not enter the tricarboxylic acid cycle. Instead it is transformed to lactate and then excreted outside the cell. It remains unclear why such rapidly dividing cells opt to waste the energetically valuable lactate in this way or why pyruvate is not processed into the tricarboxylic acid cycle to ultimately yield ATP during oxidative phosphorylation. However, it is known that the mitochondria in these cells are functional and that they primarily serve to process extracellularly derived glutamine. Indeed, this metabolism of glutamine resembles a part of the tricarboxylic acid cycle because the glutamine is converted into α-oxoglutarate and then into malate and citrate (intermediates that are then transported out of mitochondria to serve as precursors for nucleotide, lipid and amino acid synthesis). Of special interest is the fact that many cancer cells (and stem cells) express a specific isoform of pyruvate kinase, known as PK-M2. This isoform slows down the last step of glycolytic pathway, thus serving as a break to allow glycolytic intermediates to enter the pentose pathway needed for nucleotide and NADPH production [105]. This would appear to make sense, as rapidly dividing cells need to replicate their DNA before cytokinesis and hence have a high requirement for nucleotide precursors. Although in rapidly dividing, yet nonetheless healthy, cells, these adaptive responses are dependent on the presence of growth factors, cancer cells seem to have adopted various mechanisms (that I discuss below) to escape this [103].

The shift to glycolytic metabolism (Warburg effect) has been observed in non-cancerous dividing T-lymphocytes [106] and endothelial cells [107]. The proliferative response of T-cells to immune challenge is dependent on a decrease in the activity of the ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome)–Cdh1 that not only controls the G_1–S-phase transition, but also decreases glycolysis and glutaminolysis by the degradation of Prkfb3 (6-phosphofructo-2-kinase) and Gls1 (glutaminase-1) [108]. Inactivation of APC/C–Cdh1 enables T-cells to couple cell division with up-regulation of glycolysis, thus providing the lymphocytes with the components essential for the subsequent biosynthesis of macromolecules required to mount an immune response. Similarly, APC/C–Cdh1 helps post-mitotic cortical neurons to constantly down-regulate glycolysis and prevent further cell division [109].

Experimental evidence suggests that the Warburg effect seems to be even more profound in cancer cells than in the rapidly dividing, yet otherwise normal, cells of the same tissue [110]. There are many possible strategies that cancer cells can adopt to escape the tight control of their division and to become self-sufficient in their energy demands. For example, loss of PTEN (phosphatase and tensin homologue deleted on chromosome 10) (through a gene mutation) leads to high activity of Akt, subsequent activation of HIF-1α and stimulation of glycolysis. Similarly, loss of p53 or up-regulation of Oct1 enhances glycolysis, whereas up-regulation of c-Myc stimulates glutaminolysis through transcriptional regulation of enzymes in the central metabolic pathways [111,112]. It is also possible that direct mutations of the metabolic genes themselves can promote cancer, supporting the view that metabolic changes might be, at least in some cases, the cause rather than the consequence of cancer cell growth. For example, mutations in succinate dehydrogenase and fumarate hydratase in paragangliomas and phaeochromocytomas leads to the accumulation of succinate and fumarate in the mitochondria. As they leak out into the cytosol, they inhibit PHD proteins leading to the activation of HIF-1α and an accompanying glycolytic shift in metabolism [113]. Similar mechanisms are employed in brain tumours where cytosolic IDH is mutated and catalyses the production of 2-hydroxyglutarate rather than 2-oxoglutarate. As discussed above, this cancer-specific 'oncometabolite' then

inhibits the action of PHD proteins, leading to the HIF-1α-mediated development of the Warburg effect [31]. Therefore, with a growing body of experimental evidence to support him, Otto Warburg was perhaps correct, at least in part, in his assertion that "cancer can be fundamentally classified as a lesion of metabolism".

Small metabolites as regulators of cellular processes

Currently, only a handful of metabolites have been assigned a regulatory role in cell signalling. In addition to those discussed above, we can also mention steroid hormones binding to their nuclear receptors or whole range of second messengers, including phospholipids, cyclic nucleotides or arachidonic acids. However, animals generate thousands of different metabolites, without taking into account substances produced by their endosymbionts and pollutants that come from their environment [114]. It is therefore highly possible that a substantial number of metabolites harbour the potential to function as effective signalling molecules.

Notwithstanding such potential, only a few published studies have systematically assayed protein–small molecule interactions in this regard. One such study described the development of an *in vivo*-based MS assay to perform large-scale systematic analysis of hydrophobic metabolites associated with yeast proteins [115]. In this study, approximately 70% of the ergosterol biosynthetic proteins and 20% of protein kinases were found to bind hydrophobic molecules. If a percentage of that observed for the kinases were to be extrapolated to the entire proteome, then more than 1200 soluble yeast proteins would be able to interact with hydrophobic molecules [115]. Similarly, over 500 protein–lipid interactions have been discovered using an array of 56 lipids and assaying for interacting proteins [116]. Although such studies will undoubtedly carry with them a degree of false positivity with regard to biologically significance, it is still noteworthy that a substantial number of eukaryotic proteins are likely to bind as yet unknown metabolites.

Technical challenges and future perspectives

Mapping the interactions between various metabolites and other cellular components and assigning their functions undoubtedly represent an under-researched extra dimension of the cell's regulatory network that will probably form a leading direction for future research. The advance in this field is, to a large extent, dependent on the resolution of the technical challenges associated with metabolomics and proteomics. At present, combinations of MS, liquid or gas chromatography or NMR are used to detect metabolites, but, owing to their chemical diversity, it is virtually impossible to find a universal approach for the detection of all classes of cellular metabolites. Moreover, although many compounds can be detected by these methods, it remains difficult to identify their precise chemical structure. Nevertheless, it is becoming a well-accepted view that, for a given biological system, a list of metabolites could be as useful as a list of expressed genes and proteins.

Finding the interactions between proteins and metabolites and giving them a biological significance represents an even bigger challenge. Many of the interactions described so far have been identified through crystallographic studies or through the searching of protein databases for specific domain structures (e.g. the Rossmann fold or macro domain). Only a few studies have taken a purely systematic and unbiased approach to detect such interactions [115,116]. Additionally, at present, only the most basic of metabolic parameters can be measured in living cells. These are exemplified by the machines of several manufactures that are able to detect the rate of respiration or glycolysis in continuous measurements of living cells or tissues that can then be correlated with the activity of specific proteins or signalling cascades. However, genetically encoded fluorescent-protein-based sensors have been employed for FRET (fluorescence resonance energy transfer) assays, allowing the detection of several metabolites such as cAMP, cGMP, ATP, ADP or glucose in living cells or to monitor the interactions of proteins with lipids [117–120]. Other useful molecular tools in this respect are the aptamer-based riboswitch sensors [86]. Developing tools and techniques to study metabolic parameters in living cells and *in vivo* will go hand in hand with our deeper understanding of the intriguing coherence between cellular metabolism and vital cellular processes such as signalling or transcription.

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