Creatine metabolism: energy homeostasis, immunity and cancer biology

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Abstract | Perturbations in metabolic processes are associated with diseases such as obesity, type 2 diabetes mellitus, certain infections and some cancers. A resurgence of interest in creatine biology is developing, with new insights into a diverse set of regulatory functions for creatine. This resurgence is primarily driven by technological advances in genetic engineering and metabolism as well as by the realization that this metabolite has key roles in cells beyond the muscle and brain. Herein, we highlight the latest advances in creatine biology in tissues and cell types that have historically received little attention in the field. In adipose tissue, creatine controls thermogenic respiration and loss of this metabolite impairs whole-body energy expenditure, leading to obesity. We also cover the various roles that creatine metabolism has in cancer cell survival and the function of the immune system. Renewed interest in this area has begun to showcase the therapeutic potential that lies in understanding how changes in creatine metabolism lead to metabolic disease.

Creatine kinase–phosphocreatine circuit
Also known as the phosphocreatine shuttle, this system mediates the stoichiometric (1:1) transphosphorylation of phosphate from mitochondrial or glycolytic ATP to phosphocreatine, which is then used by creatine kinase to maintain high local ATP:ADP ratios.

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Endogenous creatine synthesis primarily occurs through a kidney–liver axis. Arginine and glycine are used by the enzyme glycine amidinotransferase (GATM), predominantly in the kidney, to synthesize the creatine precursor guanidinoacetate, which is exported into the circulation. Circulating guanidinoacetate is taken up by hepatocytes, which express the enzyme guanidinoacetate *N*-methyltransferase (GAMT). GAMT catalyses the second and final step of creatine synthesis using *s*-adenosyl methionine as a methyl donor. Creatine is then exported from the liver and taken up by cells that express the creatine transporter (CRT, also known as SLC6A8) (FIG. 1). In addition to this classic mechanism of creatine synthesis, other cell types, such as adipocytes, skeletal muscle cells and pancreatic acinar cells, can synthesize creatine[1–3]. Creatine can also be ingested from the diet and taken up by CRT-expressing cells, similarly to endogenously synthesized creatine.

Cellular energy demand and supply are delicately balanced. The enzyme creatine kinase catalyses the reversible transfer of a phosphoryl group from ATP to creatine (FIG. 1). There are four distinct creatine kinase isoforms — two cytosolic isoforms (CKM (creatine kinase, muscle-type) and CKB (creatine kinase, brain-type)) and two mitochondrial isoforms (CKMT1 (creatine kinase ubiquitous-type, mitochondrial) and CKMT2 (creatine kinase sarcomeric-type, mitochondrial))[4,5] — all of which are encoded by different genes. Creatine kinase isoenzymes are compartmentalized within cells to sites of ATP generation and ATP utilization. The highly ordered structure of skeletal muscle facilitated the identification of functionally coupled microcompartments, where an uneven creatine kinase distribution forms specialized associations with ATPases to use phosphocreatine to regenerate local levels of ATP, so called energy buffering[6]. The close association between intracellular energy charge (ATP:ADP ratio) and creatine biology is also observed at the level of creatine transport, where reductions in energy charge dampen creatine influx through CRT in an AMP-activated protein kinase (AMPK)-dependent manner[7].

Primarily as a result of studying creatine kinase biology in muscle, the prevailing view has been that the phosphocreatine–creatine system, together with creatine kinases that are localized at sites of ATP production (mitochondria) and consumption (such as myofibrils, sarcoplasmic reticulum and sarcolemma), can function as an intracellular energy transfer system[8]. The high degree of free energy of ATP is necessarily accompanied by low cytosolic concentrations of ADP. As diffusion requires concentration differences, the diffusion of ADP might become rate-limiting for ATP buffering. However, because phosphocreatine and creatine are closer in concentration at equilibrium than ATP and ADP, and can be present at concentrations orders of magnitude higher than ADP, the creatine kinase–phosphocreatine circuit (also known as the phosphocreatine shuttle) is well suited to balance energy supply and demand. The prevailing view

Key points

- Mitochondria in brown adipose tissue are capable of normal oxidative phosphorylation, with P:O ratios similar to those of other tissues.
- Atypical actions of creatine involve phosphocreatine transport into colorectal cancer cells, super-stoichiometric ADP liberation to trigger respiration in thermogenic adipocytes and chromatin remodelling to modulate macrophage polarity.
- Cyclocreatine and creatine can both inhibit tumour progression, suggesting that the pro-cancer role of creatine is independent of its function in energy buffering.
- The mitochondrial network transduces energy over long distances, thus minimizing the requirement for metabolite diffusion, whereas cells with a disrupted mitochondrial network might buffer energy via the creatine kinase–phosphocreatine circuit.

is that the creatine kinase–phosphocreatine circuit is primarily important in cells that are both excitable and require a high thermodynamic efficiency, such as myocytes, neurons and spermatozoa. However, the requirement for a creatine kinase–phosphocreatine circuit has also been questioned[9,10].

Many of the concepts of creatine biology have emerged from seminal studies done on creatine kinase in skeletal muscle, brain, spermatozoa and photoreceptors[11,12]. We refer the reader to expert reviews on creatine kinase function and the creatine kinase–phosphocreatine circuit[6,10–17]. New insights concerning the role of this metabolite in other cell types have developed in the past few years. Importantly, energy buffering does not seem to be the only function for creatine; indeed, new work is revealing its key role in diverse cell types and physiological conditions. This Review will focus on a subset of these functions, namely the role of creatine in adipocyte thermogenesis, immune system function and cancer cell survival.

Creatine in thermogenic adipose tissue

Adipocyte control of energy balance. Mammals have evolved to adapt to changes in energy demand and nutrient availability, facilitating their ability to occupy a diverse array of environmental niches. Adipose tissue homeostasis is under neuronal and endocrine control, giving it the plasticity required for the rapid adaptation to altered physiological states of energy demand. Shifts in the state of energy balance cause free fatty acid re-esterification (in the fed state) or triacylglycerol lipolysis (in the fasted state) to dominate, enabling the storage or mobilization of energy towards a positive or negative energy balance, respectively[18]. The development and maintenance of an appropriate quantity of adipose tissue is key for systemic metabolic health because insufficient and excess amounts of adipose tissue can lead to metabolic disease[19]. Obesity often results when energy intake chronically exceeds energy expenditure. Excess energy is primarily stored as triglycerides in adipose tissue, which expands through increases in the size (hypertrophy) and/or number (hyperplasia) of adipocytes. Energy expenditure has several major components, including basal metabolism, physical activity and adaptive (non-shivering) thermogenesis. This latter process refers to the dissipation of energy as heat that primarily takes place in brown adipose tissue (BAT) and beige adipocytes. That this process occurs most notably in response to decreased environmental temperature (cold-induced thermogenesis) and excess caloric intake (diet-induced thermogenesis) makes thermogenic adipose tissue a central regulator of thermal and body weight homeostasis[20,21].

While adipocyte thermogenesis probably evolved as a defence against hypothermia, the unique capacity for cellular thermogenesis by adipocytes could potentially be leveraged to combat obesity-associated diseases such as type 2 diabetes mellitus and fatty liver disease as well as, possibly, many cancers that are associated with these altered metabolic states[22]. BAT recruitment increases host energy expenditure and minimizes weight gain, while BAT inactivation reduces energy expenditure and can contribute to the development of obesity[23]. A reduction in the function of beige adipose tissue can also lead to obesity and insulin resistance[24]. Over the past decade, several studies have definitively shown that adult humans have BAT and that its activity can account for up to 5% of the basal metabolic rate, which, if BAT is activated, could promote more than 4 kg of adipose tissue loss per year[25–27].

UCP1-independent thermogenesis. UCP1 is an effector protein of thermogenic respiration in brown adipocytes (FIG. 2). It is a mitochondrial inner membrane protein that dissipates the proton gradient across the lipid bilayer, effectively decreasing the proton-motive force and minimizing ATP synthesis. Consequently, the energy dissipated across the mitochondrial inner membrane results in a considerable increase in the rate of respiration, substrate oxidation and release of heat. When bred on a congenic background, *Ucp1*$^{-/-}$ mice become hypothermic upon acute cold exposure; however, they can survive the cold if they are gradually adapted to these lower environmental temperatures[28–31]. The chief argument, by some, for UCP1 being the only thermogenic effector is as follows: as *Ucp1*$^{-/-}$ mice do not cease to shiver in the cold, no alternative thermogenic pathway (or pathways) can be of sufficient physiological relevance because the requirement for shivering is not abolished[32]. The assumption of this argument is that muscle shivering is the sole mediator of thermal homeostasis in cold-adapted *Ucp1*$^{-/-}$ animals. However, first, there is no experimental evidence to support this conjecture. Second, shivering cannot fully explain the ability of these mice to adapt to slow cold exposure because the degree of shivering in *Ucp1*$^{-/-}$ mice that are acutely or slowly exposed to cold is quantitatively and qualitatively identical[28]. Third, and most importantly, *Ucp1*$^{-/-}$ animals on a hybrid background maintain body temperatures indistinguishable from those of wild-type animals even following acute cold exposure[33]. Thus, UCP1 is dispensable for cold-mediated thermogenesis.

Another important consideration that cannot be overlooked relates to our identification that levels of most components of the electron transport chain in BAT of constitutive *Ucp1*$^{-/-}$ mice are greatly reduced compared with components in BAT from wild-type mice[34]. This global mitochondrial dysfunction is evident at thermoneutrality and becomes incredibly striking at sub-thermoneutral temperatures (when many phenotypes of *Ucp1*$^{-/-}$ brown adipocytes become apparent)[34,35].

UCP1
A mitochondrial inner membrane protein that dissipates the proton gradient across the lipid bilayer, effectively decreasing the proton-motive force and minimizing ATP synthesis; the energy dissipated across the mitochondrial inner membrane results in a considerable increase in the rate of respiration, substrate oxidation and release of heat.

Proton-motive force
The potential energy stored as a combination of the electrical and concentration (electrochemical) gradient across the mitochondrial inner membrane due to the extrusion of protons into the intermembrane space by the electron transport chain.

Congenic background
An inbred strain of mouse where the control and experimental animals only differ from one another by a small genetic region (typically a single gene).

Thermoneutrality
The ambient temperature where the metabolic rate is at a minimum, when temperature regulation is achieved by non-evaporative physical processes alone.

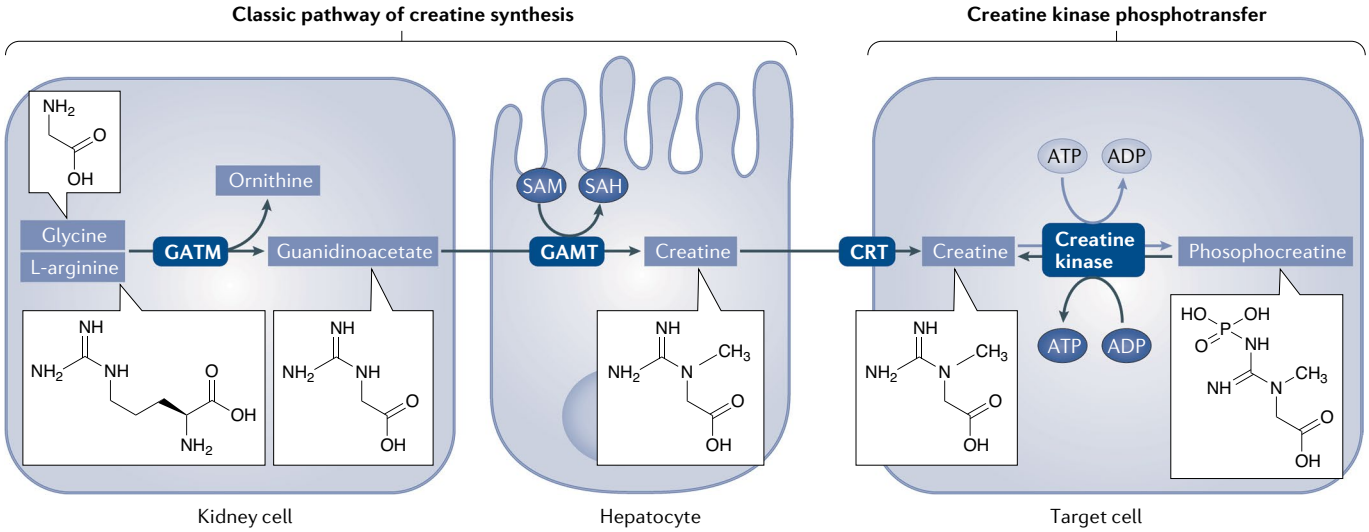

Fig. 1 | **Pathway of endogenous creatine synthesis.** In the kidney, glycine amidinotransferase (GATM) catalyses the synthesis of guanidinoacetate. Guanidinoacetate is exported to the circulation and taken up by hepatocytes, where guanidinoacetate *N*-methyltransferase (GAMT) converts it to creatine. Creatine is effluxed from hepatocytes into the circulation. Creatine is sequestered by cells expressing the creatine transporter (CRT). Creatine can be phosphorylated to phosphocreatine by creatine kinase, using ATP. During times of high and fluctuating energy demand (high ATP consumption), phosphocreatine can be used in reverse by creatine kinase to buffer the ATP pool. SAH, *s*-adenosylhomocysteine; SAM, *s*-adenosylmethionine.

Consistent with this finding, the respiratory rate of *Ucp1*$^{-/-}$ BAT mitochondria is greatly decreased in response to chemical uncoupling, particularly following cold exposure[36,37]. With these data, a reasonable conclusion is that the *Ucp1*$^{-/-}$ mouse model exhibits defects in BAT beyond UCP1. Consistent with this supposition, mitochondria from BAT of *Ucp1*$^{-/-}$ mice are extraordinarily sensitive to dysfunction induced by calcium overload[34]. Investigators, including ourselves, that have used these *Ucp1*$^{-/-}$ mice must interpret their data in light of these findings. However, despite the knowledge that the phenotype of *Ucp1*$^{-/-}$ mice cannot be attributed entirely to UCP1, these mice are potentially useful for identifying alternative thermogenic pathways in brown and beige adipocytes. Nevertheless, we would argue that alternative pathways that only get activated upon *Ucp1* inactivation are probably of limited physiological relevance. If physiologically relevant, genetic manipulation of components regulating these alternative pathways should reveal phenotypes related to energy balance and/or nutrient homeostasis without the need for genetic inactivation of *Ucp1*.

The most unexpected phenotype of *Ucp1*$^{-/-}$ mice is their normal resting energy expenditure and resistance to weight gain on a high-fat diet under standard room temperature housing conditions (~20–24°C is the assumed housing temperature, unless otherwise noted)[38]. This was an unforeseen observation given the involvement of BAT in diet-induced thermogenesis[39] and the idea that UCP1 was the primary effector of diet-induced thermogenesis. Subsequently, it was proposed that thermoneutral housing was required to reveal the effect of UCP1 on mitigating obesity[40]; however, this might not be the case, as several reports have now demonstrated that obesity is not apparent in *Ucp1*$^{-/-}$ mice, even when housed at thermoneutrality[41–44]. Some investigators interpret these data to mean that diet-induced thermogenesis is of little physiological relevance to body weight control. However, it is equally plausible that diet-induced thermogenesis is a robust mechanism to offset weight gain from overfeeding and that UCP1 is simply not the effector of this process. Indeed, mice with targeted BAT ablation (UCP-DTA mice) develop diet-induced obesity to a greater extent than control mice with functional BAT[23]. Obesity develops in UCP-DTA mice in the absence of hyperphagia, which indicates that these mice have an increased metabolic efficiency. However, hyperphagia does develop, but only after obesity has already been established[23]. Similarly, mice lacking all three β-adrenergic receptors (β-less mice) develop substantial obesity on a high-fat diet at room temperature[45], again without any change in energy intake. β-Adrenergic receptors act via the second messenger cyclic adenosine monophosphate to trigger a signalling cascade that stimulates the mobilization of stored triacylglycerol into free fatty acids to support thermogenesis[46]. How can two distinct models with defective BAT (UCP-DTA and β-less) reveal such different phenotypes from that of *Ucp1*$^{-/-}$ mice under similar conditions? A reasonable conclusion is that additional thermogenic effectors, downstream of β-adrenergic signalling, are still operational in these animals. These thermogenic mechanisms cannot be fully recruited in the context of BAT diminution itself (UCP-DTA) or through inactivation of adrenergic signalling all together (β-less)[23,45,47,48].

It has been suggested that *Ucp1*$^{-/-}$ mice are protected from diet-induced obesity, particularly at subthermoneutral temperatures, because they must induce alternative thermogenic pathways that are less efficient

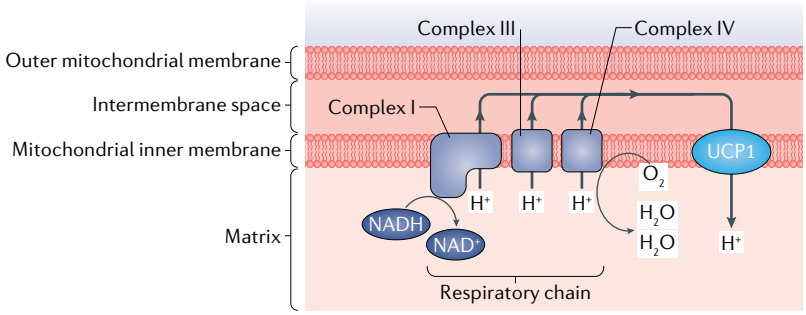

Fig. 2 | **UCP1-dependent thermogenesis.** The respiratory chain pumps protons into the intermembrane space, generating an electrochemical gradient across the mitochondrial inner membrane. Thermogenic mechanisms trigger an increase in the rate of substrate oxidation, which leads to thermogenesis. UCP1 dissipates the energy stored in this gradient by mediating H^+ leak from the intermembrane space to the matrix.

than the UCP1 pathway (they require more calories to produce the same amount of heat) to sustain their body temperature[49]. Consistent with this idea, *Ucp1*$^{-/-}$ mice can still activate ~50% of cold-mediated heat production compared with wild-type animals[29,30,41,50,51]. However, just as UCP1 is apparently equally thermogenic in beige adipose tissue and BAT[52], there is no reason to assume that BAT cannot operate UCP1-independent thermogenic pathways, as has been proposed for beige fat[30,50,53]. Indeed, using magnetic resonance thermometry with hyperpolarized xenon, the direct detection of BAT-mediated thermogenesis was shown to occur in inbred *Ucp1*$^{-/-}$ mice[54]. The BAT temperature of *Ucp1*$^{-/-}$ mice increases following noradrenaline administration, which precedes a rise in rectal temperature, corroborating the idea that UCP1-independent thermogenic pathways are operational in classic BAT[54].

One study has reported that UCP1 can combat obesity during ageing[55]. It is well known that the amount of BAT is inversely correlated with age[56,57]. The development of obesity in *Ucp1*$^{-/-}$ animals upon ageing is unexpected given that the difference in UCP1 levels between wild-type and *Ucp1*$^{-/-}$ mice should be greatest in young animals as they have greater amounts of BAT than old animals[58]. A similar argument can be put forth regarding the report that thermoneutral housing is needed to reveal the obese phenotype of *Ucp1*$^{-/-}$ mice[40] as larger differences in levels of UCP1 between wild-type and *Ucp1*$^{-/-}$ mice occur at sub-thermoneutral housing temperatures. If thermoneutral housing and ageing are required to trigger obesity in *Ucp1*$^{-/-}$ mice, this suggests that these environmental conditions must impair UCP1-independent anti-obesogenic biochemical pathways because *Ucp1* deletion on its own is not sufficient to trigger obesity. The concomitant impairment of these thermogenic pathways along with *Ucp1* deletion might then trigger obesity.

As a result of the impairment of BAT mitochondrial biology in *Ucp1*$^{-/-}$ mice, the physiological relevance of UCP1-independent pathways to thermogenesis might be underestimated if these mice are used to identify such pathways. Pathways that are not mediated by UCP1 need to be studied in isolation, with intact UCP1 function, to reveal their relevance. Indeed, the physiological relevance of creatine metabolism in adipose tissue is demonstrable by the fact that *Ucp1* inactivation is not necessary to reveal its role in thermogenesis[2,53,59–65]. Reducing levels of creatine in adipocytes is sufficient on its own to impair the activation of energy expenditure in response to pharmacological β_3-adrenergic activation or high-fat feeding (diet-induced thermogenesis)[2,53,61]. Similarly, global inactivation of creatine transport causes elevated adipose accumulation[66]. These phenotypes are observed in housing conditions associated with some or no beige adipose tissue, indicating that creatine-dependent thermogenesis is probably operational both in beige adipose tissue and BAT. Genetic and pharmacological depletion of creatine-dependent thermogenesis often results in elevated levels of UCP1 and vice versa (but not always)[2,34,53]. Nevertheless, compensatory relationships between genes encoding the components of the creatine synthesis and transport pathway and UCP1 suggest that these gene products constitute the components of parallel thermogenic pathways. These data demonstrate that, when one thermogenic pathway is inactivated, the other pathway is triggered to mitigate any thermogenic impairment. Similar reciprocal relationships occur with UCP1 in beige adipose tissue following surgical BAT ablation[67] and with UCP1 in BAT following inactivation of non-adipose thermogenesis[68]. The next sections will examine how a cell can generate heat independently of UCP1.

Creatine-dependent thermogenesis. Early in vivo creatine uptake experiments demonstrated that radioactive labelling of the creatine pool with [^{14}C]creatine proceeded more rapidly in BAT than in skeletal muscle[69]. Brown adipocytes have creatine kinase activity in the same order of magnitude as cardiac or nerve tissue[69]. However, the precise creatine kinase isozyme responsible for most of this activity is currently unknown. The two mitochondrial creatine kinases, CKMT1 and CKMT2, are enriched in human BAT compared with white adipose tissue at the mRNA and protein level[59,64,70], and the genes encoding these kinases are co-ordinately regulated with *UCP1* (REF.[64,71]).

In adipocytes, creatine energetics are a key regulator of BAT thermogenesis[2,59–65], with creatine in adipocytes coming from both intracellular synthesis and sequestration from the circulation[2,61]. Genetic depletion of creatine levels in adipocytes through either inhibition of synthesis or uptake from the circulation impaired adipocyte thermogenesis and potentiated diet-induced obesity[2,61], recapitulating the elevated adipose tissue accumulation that occurred in animals with global inactivation of creatine transport[66]. The forward and reverse phosphotransfer reactions of phosphocreatine–creatine in most cells occur in 1:1 stoichiometry with the ATP–ADP couple. However, mitochondria in thermogenic adipocytes seem to liberate a molar excess of ADP with respect to creatine, as concluded by observing the respiratory response to creatine under ADP-limited conditions[53,60]. Thus, in thermogenic adipose, creatine triggers a substrate cycle of mitochondrial ADP liberation in a super-stoichiometric manner. This futile creatine cycle is understood to be triggered by

Creatine-dependent thermogenesis
The phosphorylation of creatine by creatine kinase and subsequent dephosphorylation of phosphocreatine (or downstream phosphometabolite) that regenerates creatine and dissipates the high-energy phosphate to generate heat; also known as futile creatine cycling.

phosphorylation of creatine by creatine kinase-mediated phosphotransferase activity and a phosphatase that hydrolyzes phosphocreatine to regenerate creatine[53,60]. This pathway has been expertly reviewed elsewhere[22], so here we will focus on the latest investigations that have examined the association of creatine biology with energy expenditure.

The expression of CKMT2 is highly elevated in two mouse models that have substantial reductions in adipose tissue accumulation in response to caloric excess[72,73]. As in most G protein-coupled receptors, the activity of adrenergic receptors is terminated by their agonist-induced internalization by β-arrestins[74]. In adipose tissue, β-arrestin 2 (encoded by *Barr2*) is a strong negative regulator of adipocyte β_3-adrenergic signalling. Stimulation of the β_3-adrenoreceptor is followed by its robust *Barr2*-mediated internalization[72]. Mice lacking *Barr2* selectively in adipose tissue were protected from diet-induced obesity and exhibited substantial activation of mitochondrial creatine kinase activity in adipocytes, which was associated with large increases in *Ckmt2* mRNA and CKMT2 protein levels[72]. Thus, it is likely that futile creatine cycling has a key role in mediating the beneficial metabolic phenotypes exhibited by these animals. Similarly, the selective activation of G_s signalling in adipose tissue caused a profound activation of energy expenditure, reduced weight gain upon feeding with a high-fat diet, and resulted in improvements in glucose tolerance and insulin sensitivity[73]. These beneficial effects driven by adipocyte thermogenesis were also associated with considerable increases in *Ckmt2* expression as well as with increases in the expression of other genes associated with thermogenesis (*Ucp1*, *Pgc1α* and *Prdm16*). Together, these data confirm prior work[60] demonstrating that genes with protein products involved in creatine metabolism are downstream of canonical adrenergic signalling in adipose tissue.

New evidence strongly supports the idea that PGC1α (a transcriptional co-activator) coordinates the activation of creatine-dependent thermogenesis and that disruption of this pathway impairs energy expenditure and potentiates diet-induced obesity[75]. Specifically, PGC1α acetylation by *N*-α-acetyltransferase 10 (NAA10) blocked the interaction between PGC1α and PPARγ and suppressed thermogenesis[75]. Mechanistically, the acetyltransferase activity of NAA10 inhibited PGC1α-dependent transcriptional regulation, while deletion of NAA10 or an acetylase-dead mutation enhanced PGC1α occupancy on promoters of genes with protein products involved in thermogenesis, such as *Ckmt2*. The adipose tissue-selective deletion of NAA10 in mice increased energy expenditure, mitigated diet-induced obesity and increased the expression of genes associated with thermogenesis, such as *Ckmt2* and *Ucp1* (REF.[75]).

The adipose tissue-selective deletion of CRT (encoded by *Slc6a8*) in mice (AdCrTKO) reduces creatine and phosphocreatine abundance in BAT and results in mild cold intolerance without a change in the abundance of UCP1 in BAT[61]. Along with impaired thermoregulation, AdCrTKO animals exhibit suppressed thermogenesis following β_3-adrenergic agonism. Short-term feeding with a high-fat diet in wild-type mice induces CRT protein expression in mature brown adipocytes, which is correlated with elevated creatine influx into BAT. Importantly, the ability to activate diet-induced thermogenesis is blunted in AdCrTKO mice, which is associated with increased metabolic efficiency and susceptibility to diet-induced obesity[61]. Consistent with this finding, independent work has shown that mice with global deletion of CRT exhibit similar levels of creatine depletion as AdCrTKO animals and have increased body adipose tissue stores compared with controls[66]. Finally, *SLC6A8* expression in human subcutaneous adipocytes is negatively correlated with BMI and insulin resistance[61], suggesting that futile creatine cycling might be relevant in humans.

Ependymin-related protein 1 (EPDR1) has been identified as a factor secreted by brown adipocytes and not by white adipocytes, suggesting that it regulates thermogenic respiration[76]. BAT from *Epdr1*$^{-/-}$ mice exhibited reduced oxygen consumption in the basal state, without alteration in UCP1 abundance, and these mice gained considerably more adipose tissue than controls. Specifically, EPDR1 depletion in human brown adipocytes impaired adrenergic-dependent respiration and was associated with a statistically significant reduction in the levels of CKMT1 at the protein and mRNA level[76]. These data are consistent with creatine energetics having an important thermogenic role in human adipose tissue.

Humans express four isoforms of ADP/ATP translocase (ANT; ANT1–ANT4), while mice lack ANT3 and mainly express ANT1 and ANT2 (REF.[77]). Creatine-mediated thermogenesis requires the exchange of mitochondrial ATP for cytosolic ADP through ANT[53]. This feature of creatine-dependent respiration agrees with the established functional coupling of AAC isoforms with mitochondrial creatine kinase isoforms[15,78]. Chemical inhibition of all ANT isoforms blocks ADP-dependent respiration and thus impairs futile creatine cycling. Mice with adipocyte-specific knockout of ANT2 (ANT2 AKO) exhibit increased visceral adipose tissue mass and adipocyte hypertrophy, concomitant with an increased respiratory exchange ratio, indicating a reduced reliance on lipid-derived metabolic fuels[79]. Visceral adipocytes from ANT2 AKO mice exhibit decreased oxygen consumption following the consumption of a high-fat diet compared with adipocytes from control animals, suggesting that ANT2 might support diet-induced thermogenesis[79].

The mechanism for the elevation in oxygen consumption following overfeeding was proposed to be caused by ANT2-mediated uncoupling as inhibition of ATP synthesis by administration of oligomycin did not entirely ablate differences in basal oxygen consumption. Indeed, ANT1 and ANT2 control oxygen consumption under conditions where mitochondrial ATP synthesis is blocked[80], suggesting that H^+ leak by all ANT isoforms is inhibited by their ADP/ATP carrier activities. Importantly, using whole mitochondrial inner membrane patch-clamping, the first direct evidence of ANT-mediated H^+ leak was demonstrated in non-UCP1-expressing mitochondria, including those from skeletal muscle, liver, kidney and heart[81].

The relative contribution of ANT2-mediated uncoupling and ADP–ATP transport to thermogenesis in these cells merits further investigation. Moreover, it is now known that both ANT1 and ANT2 control fatty acid-mediated H^+ leak respiration. Whether some of the phenotype of the ANT2 AKO mice is masked by ANT1 compensation remains to be investigated. It will be of interest to determine whether the phenotype of animals with adipose-specific double knockout of ANT1 and ANT2 phenocopies mice with creatine depletion in their adipocytes.

Mice with a dual germline deletion of *Ckb* and *Ckmt1* in all tissues (*Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$) exhibit defective thermoregulation without alteration in UCP1 abundance[82]. The mechanism underlying this thermal impairment was proposed to be due to diminished neuronal transmission. However, *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ animals display several defects that could influence body temperature independently of neuronal function, such as reduced body weight and decreased muscle and liver mass[83]. These effects are probably caused by decreased food intake, as *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice consume ~15% less total food per day than control animals[82]. Chronic reductions in food consumption of this magnitude can easily explain the reduced body weight of *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice. In addition, because thermoregulation is related to food intake, it is possible that the reductions in daily food intake predispose *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice to impaired thermoregulation. *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice display chronically elevated activity levels compared with control mice, which would further contribute to a negative energy balance[82].

Interestingly, the temperature of BAT was reduced in *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice compared with that of control animals following noradrenaline administration, suggesting that the function of BAT might be impaired specifically in *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice[82]. However, although BAT certainly generates heat, increased tissue temperature does not necessarily indicate that this is caused by increased metabolism[84]. Differences in blood flow and vasoconstriction could influence tissue temperature without changing thermogenesis. For now, it is not possible to confidently determine whether the defective thermoregulation of *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice is specific to any single tissue or is a secondary consequence resulting from multi-tissue dysfunction and altered caloric intake. To examine whether impaired neuronal transmission is causative for defective thermoregulation, brain-specific deletion of *Ckb* and *Ckmt1* is required. The possibility that the defective thermoregulation of *Ckb*$^{-/-}$/*Ckmt1*$^{-/-}$ mice is partly attributable to ablated futile creatine cycling in adipose tissue is still an open question. Adipose tissue-specific deletion of *Ckb* and *Ckmt1* will be required to test their role in adipocyte biology.

Futile creatine cycling: important considerations. Current data in support of the model of futile creatine cycling suggest two possibilities: first, and most simple, a pool of intra-mitochondrial phosphocreatine and creatine circulating within the intermembrane space (generated by mitochondrial creatine kinase and a phosphocreatine phosphatase, respecvitely) or, second, channelling of phosphocreatine out of the mitochondrial intermembrane space towards phosphocreatine phosphatase activity outside the organelle (FIG. 3a). Mitochondria make contact sites with other organelles, such as the endoplasmic reticulum (ER), and these contact sites are maintained during standard mitochondrial purification techniques. As creatine and phosphocreatine can readily pass through the outer mitochondrial membrane via the voltage-dependent anion channel (VDAC), it is plausible that phosphocreatine is hydrolysed following its release, possibly at mitochondria–ER contacts (FIG. 3a). After phosphocreatine is hydrolysed, the regenerated creatine could then easily diffuse back through VDAC into the mitochondrial intermembrane space to stimulate a further round of ADP-dependent respiration. This topology and ease of creatine diffusion could drive recurrent energy dissipation. Substrate channelling through mitochondrial–ER contact sites via VDAC as well as the formation of these contact sites themselves could provide the required tight regulation of phosphatase activity to avoid excessive depletion of the cytosolic energy state (low phosphocreatine to ATP ratio). Considering that mitochondria undergo fission during thermogenic respiration[85] and the fact that ER tubules dictate the site of mitochondrial division[86], this possibility seems worthy of investigation.

Any biochemical reaction that occurs in vivo in a living system does not occur in isolation but is part of a metabolic pathway, which makes conceptualizing the relationship between reactants and reactions challenging. Enzyme kinetic measurements (K_{cat} and K_m) characterized in vitro cannot simply be extrapolated to the in vivo environment[87]. If the physiological concentration of a substrate is lower than the K_m of the enzyme acting on that substrate, then the activity of that enzyme will largely depend on substrate availability. This feature might be particularly important for futile creatine cycling as the super-stoichiometric action of creatine in intact mitochondria is elicited with concentrations of creatine below the apparent K_m of purified mitochondrial creatine kinase[53,60,88]. However, in a native environment, enzyme activity can be markedly different compared with that of purified proteins[89,90]. These differences could be due to intracellular allosteric effectors, binding partners, post-translational modifications, crowding and microcompartments that would be lost from work done with purified proteins in isolation. To our knowledge, the physiological concentration of creatine in thermogenic adipocytes (in culture or in vivo) has never been examined using quantitative methods with isotopic internal standards. Using these quantitative methods will be a key consideration as this area of creatine research moves forward.

Mitochondria purified from BAT have long been known to be capable of normal oxidative phosphorylation in the presence of endogenous purine nucleotide levels and when fatty acids are controlled by addition of bovine serum albumin to mimic intracellular fatty acid carrier proteins[91–93]. As adipocytes contain high levels of fatty acids, fatty acids will uncouple mitochondrial respiration to an extent not elicited in normal physiology. Thus, the physiological function of mitochondria

is better represented following their purification in the presence of bovine serum albumin. The liberation of fatty acids in brown adipocytes can partly uncouple respiration from ATP synthesis. However, it is critical to note that a substantial portion of adrenergically stimulated brown adipocyte respiration is inhibited by oligomycin (an ATP synthase inhibitor)[94] and BAT mitochondria from cold-adapted rats and rabbits are capable

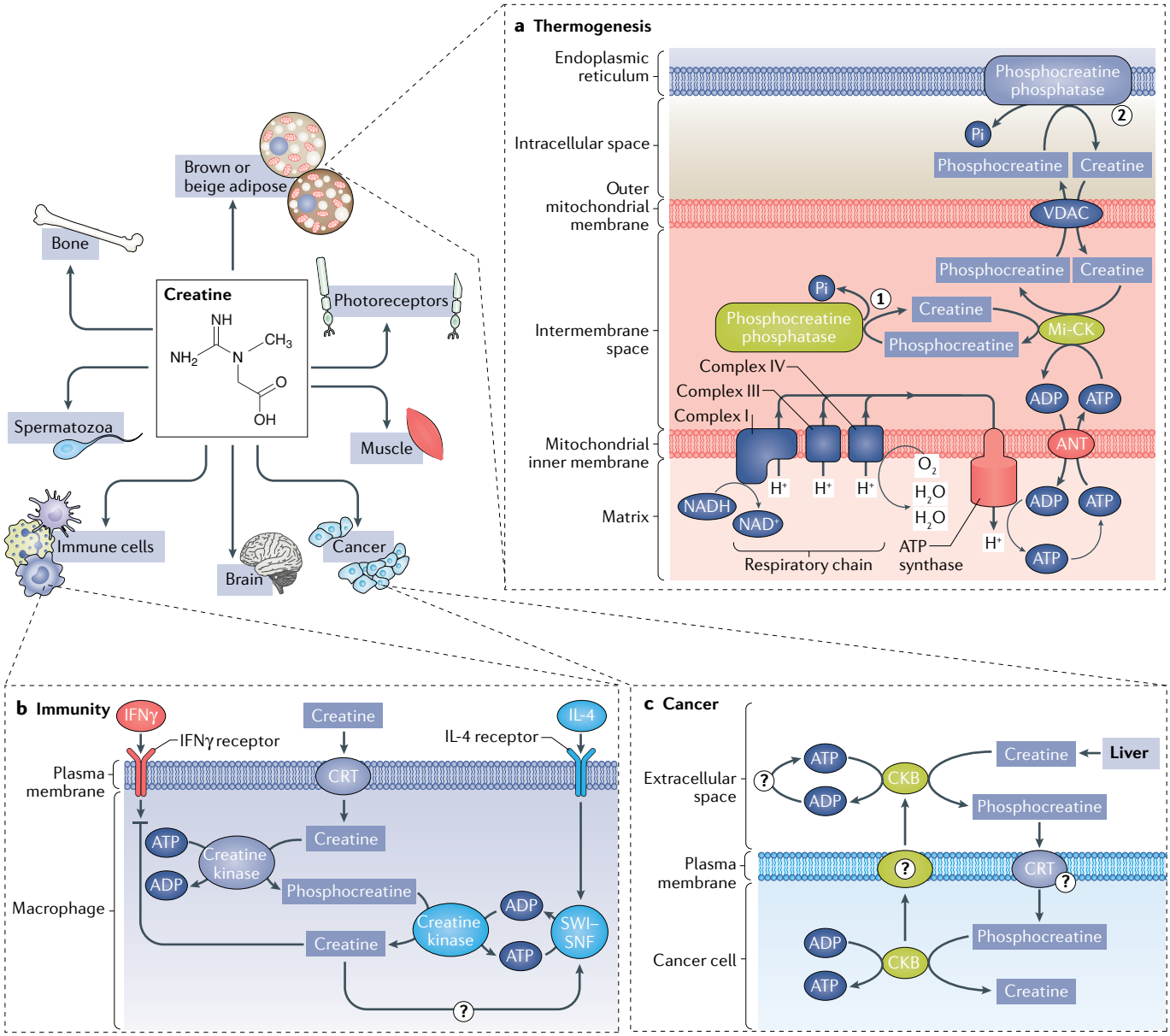

Fig. 3 | **Creatine biology in various cell types.** Creatine biology has been mainly studied in skeletal muscle, spermatozoa, brain and photoreceptors, with creatine playing a key role in energy buffering in these cells. However, creatine also has key roles in brown and beige adipose tissue thermogenesis, immune function and cancer cell survival. Creatine dissipates the energy stored across the mitochondrial inner membrane by stimulating a cycle of ATP turnover, mediated by mitochondrial creatine kinase and coupled to the turnover of phosphocreatine (part **a**). This model of phosphocreatine turnover can accommodate direct hydrolysis by a phosphocreatine phosphatase or a downstream phosphatase that mediates phosphate hydrolysis from another metabolite following several phosphotransfer reactions that start with phosphocreatine. The hydrolysis of phosphocreatine might occur within mitochondria (pathway 1, white circle) or within organelles that contact mitochondria (pathway 2, white circle), such as the endoplasmic reticulum. Creatine is taken up by macrophages to modulate macrophage polarity (part **b**). Creatine inhibits IFNγ-dependent proximal signalling to mitigate activation of the M1-like phenotype. The block on M1 signalling seems to be independent of classic creatine-dependent energy buffering. Conversely, creatine is hypothesized to promote the M2-like phenotype by facilitating ATP-dependent chromatin remodelling by the SWI–SNF complex. Whether classic creatine energetics fully explain the actions of creatine on M2-like polarization remains to be established. Creatine kinase brain-type (CKB) is secreted from colorectal cancer cells to transphosphorylate creatine into phosphocreatine in the extracellular milieu using extracellular ATP (part **c**). Phosphocreatine is then utilized by colorectal cancer cells for energy buffering to support survival in the liver microenvironment. The mechanism of CKB secretion is unknown and the regulation and mechanism of phosphocreatine influx by the annotated creatine transporter (CRT) remains to be determined. A source of extracellular ATP is required to maintain phosphocreatine-mediated cancer cell survival. The precise source for this ATP is not completely understood. ANT, ADP/ATP translocase, Mi-CK, mitochondrial creatine kinase; VDAC, voltage-dependent anion channel.

P:O ratio
The number of moles of ADP phosphorylated to ATP for every two electrons that reduce oxygen to water.

of oxidative phosphorylation with a P:O ratio similar to those of other tissues[92]. Thus, an important and often overlooked aspect of brown adipocyte function is that the coupling apparatus of their mitochondria is unusually labile and not as easily demonstrable in vitro as that of other tissues. However, mitochondria isolated under proper conditions from the BAT of cold-adapted rats clearly exhibit oxidative phosphorylation that can be uncoupled by the protonophore 2,4-dinitrophenol[91].

Human BAT has also been demonstrated to participate in both coupled and uncoupled respiration[59]. In non-adrenergically stimulated cells, brown adipocyte respiration is primarily controlled by ATP demand[95]. ATP seems to take on a larger role of supporting fatty acid oxidation during adrenergic activation of adipose tissue based on the ability of oligomycin to impair both noradrenaline-mediated and protonophore-mediated activation of respiration[96]. However, noradrenaline-stimulated respiration in brown adipocytes is highly variable (depending on the buffer conditions used for respirometry) and so it is conceivable that in vivo conditions might still allow for the utilization of coupled respiration during adrenergic stimulation[97]. Indeed, inhibition of the ANT inhibits noradrenaline-dependent respiration when noradrenaline is used at limiting amounts, which indicates that oxidative phosphorylation is operational in adrenergically stimulated brown adipocytes[97]. It has been proposed that substrate level phosphorylation from the citric acid cycle is responsible for providing the ATP required for fatty acid oxidation during adrenergically stimulated respiration[94]. Nevertheless, there are now several new pathways that stimulate BAT respiration independent of the adrenergic–lipolytic cascade[98,99]. These adrenergic-independent mechanisms of thermogenic stimulation might be relevant for thermogenic pathways that have the flexibility to utilize metabolic fuels distinct from fatty acids.

The pertinent question is, which conditions favour coupled versus uncoupled respiration in vivo? Purine nucleotides, primarily ATP in vivo, can inhibit UCP1 by binding to the cytosolic side of the protein. BAT takes up fatty acids from the circulation, which are released following cold-mediated activation of the lipolytic cascade in white adipocytes[100,101]. These fatty acids compete with the purine nucleotide, binding to UCP1 and supporting the re-entry of protons into the mitochondrial matrix. Clearly, cold exposure is a powerful stimulus that drives catabolic processes as well as the loose coupling of oxidative phosphorylation, which is an efficient means of heat generation. In contrast to cold-mediated thermogenesis, BAT also participates in diet-induced thermogenesis, which occurs in the fed state when energy reserves are replete. Thus, ATP levels would not be reduced under these conditions and such a physiological situation might stimulate the thermogenic pathways that can primarily use ATP as a sink. In this scenario, ATP is not likely to be limiting and is more prone to be utilized for energy-dissipating biochemical pathways. New therapeutic treatments for metabolic diseases targeting adipocyte dysfunction might benefit from a refined understanding of creatine biology in adipose tissue.

Creatine in thermogenic adipose tissue: future perspectives. An argument could be put forth that any alternative thermogenic pathway that only attains relevance upon UCP1 deletion is still, in fact, dependent on UCP1 (inactivation). Deletion of *Ucp1* is not required for all non-UCP1 thermogenic pathways to reveal their functional role in supporting energy homeostasis in response to physiological challenges such as cold exposure and caloric excess[2,61,102,103]. Creatine-dependent thermogenesis seems to fall into this category of UCP1 independence. Moreover, the compensatory nature of UCP1 abundance and the expression of genes associated with creatine transport and metabolism also points to futile creatine cycling as being a parallel thermogenic pathway to the UCP1 pathway[2,34,53]. While this finding certainly highlights the physiological relevance of creatine metabolism in thermogenic adipose tissue, creatine metabolism might also somehow alter brown adipocyte function to support UCP1-mediated thermogenesis. However, no experimental evidence thus far suggests that this is the case. The current data indicate that thermogenic respiration mediated by creatine and UCP1 are both operational independent of one another. How might we envisage that two thermogenic pathways, essentially requiring the extraction of energy stored in the mitochondrial proton-motive force, interact within a BAT depot? One possibility is that these two pathways occur in different adipocyte subtypes within BAT, which is known to be heterogenous[104]. Another possibility is that futile creatine cycling is favoured in the acute phase (for example, following acute transition to environmental cold) until UCP1 levels accumulate to sufficiently high levels to take over thermogenic function. Alternatively, both pathways might be operational in the same cells but be preferentially utilized under distinct nutritional states (such as UCP1 for cold and creatine for diet).

The molecular mechanisms regulating futile creatine cycling downstream of creatine accumulation in adipocytes are not fully understood. Mechanistically, creatine-dependent thermogenesis can trigger mitochondrial ADP liberation, which is understood to depend on the phosphorylation of creatine by creatine kinase-mediated phosphotransferase activity[53,60]. However, whether creatine kinase activity primarily regulates ADP liberation to drive thermogenic respiration[53,60] or whether classic creatine-dependent spatiotemporal buffering of ATP[6,15] is also operational in thermogenic adipose tissue is not known. Moreover, the key creatine kinase isozyme (or isoenzymes) and the proposed phosphocreatine phosphatase that control thermogenic respiration in adipocytes have yet to be identified (FIG. 3a). Finally, the extrinsic thermogenic stimuli that regulate creatine metabolism in adipocytes are currently unknown, but will be an important area of future investigation around signals that trigger futile creatine cycling.

Creatine in immunity

T cells. T cell development in the thymus is a key step in the establishment of a properly functioning adaptive immune system. Bone marrow-derived T cell precursors migrate to the thymus, where the cells' genes that encode

the T cell receptor (TCR) are rearranged and their fates are dictated[105]. Owing to the rearrangements of numerous gene segments, the TCR repertoire can reach a high combinatorial diversity[106]. The affinity of the TCR for self-peptide–MHC complexes expressed on antigen-presenting cells is the crucial parameter that drives developmental outcomes in the thymus. For developing T cells, many of the randomly reshuffled TCRs do not function because they do not bind host MHC alleles. Thymocytes with no or very low TCR-mediated affinity for self-peptide–MHC complexes undergo apoptosis (death by neglect). A key step in T cell development is positive selection, which enriches for T cell progenitors that are MHC restricted by allowing only cells that express a TCR that can interact with self-peptide–MHC complexes to differentiate further. This step also enriches for self-reactive cells, thereby making the risk of autoimmunity inherent in the adaptive immune system. To limit this risk, the most strongly self-reactive progenitors are subject to negative selection and die locally in the thymus, thus being eliminated from the T cell pool. Accordingly, it is primarily the weakly reactive progenitors that mature, populate the lymphoid organs and participate in immune responses to foreign antigens[107].

Creatine is emerging as a key player in immune cell function[14]. CKB regulates thymocyte selection and T cell proliferation and activation by modulating TCR signal strength. Specifically, double positive (DP; $CD4^+$ and $CD8^+$) thymocytes have fairly low expression levels of CKB but, following interaction with self-antigens and selection, single positive $CD4^+$ T cells and $CD8^+$ T cells strongly upregulate CKB abundance[108]. The activation, proliferation and cytokine secretion of T cells were enhanced by the expression of a *Ckb* transgene under the control of CD2-based regulatory elements that conferred ectopic expression of *Ckb* in the T cell lineage. Notably, transgenic *Ckb* expression decreased the number of cells in the thymus by half, which was primarily driven by a decrease in DP cells. The decrease in thymus cells was shown to be mediated by CKB-mediated hyperactivation of DP cells, followed by apoptotic negative selection in the thymus in vivo[108]. *Ckb* overexpression augmented the apoptosis of DP thymocytes following mimicry of negative selection in vitro with anti-TCR and anti-CD28 activation. By contrast, when T cells were activated in a similar manner, the proliferative capacity and activation of *Ckb*-overexpressing T cells was higher than in controls, while treatment of T cells with Ckb short hairpin RNA or cyclocreatine (a creatine analogue that is a poor substrate for the energy buffering activity of creatine kinase) resulted in severely impaired T cell activation[108]. These data point to CKB as a key regulator of T cell development and activation through the control of TCR signalling. How might this occur? CKB has been proposed to amplify TCR signalling by supporting the increased levels of ATP required for kinase-mediated signalling. This proposal suggests that ATP levels might be limiting for full activation of T cells, at least in vitro. Indeed, CKB overexpression was associated with increased ATP levels (corresponding levels of ADP, creatine or phosphocreatine were not reported)[108]. Thus, future work in this area should explore whether CKB overexpression alone can shift the creatine kinase phosphotransfer equilibrium towards ATP production. If this is the case, one would expect a reduction in phosphocreatine of a similar magnitude. Whether creatine kinase activity also regulates signalling pathways, such as cytokine production, certainly merits further investigation.

Thymocyte selection
During T cell differentiation, thymocytes can undergo expansion, differentiation (positive selection) or cell death (negative selection).

Macrophages. In tissues, macrophages are important members of the mononuclear phagocyte system, where they regulate adaptive immune responses to invading pathogens[109]. Changes in cellular metabolism modulate macrophage function through a process termed polarization. When exposed to IFNγ and/or lipopolysaccharide, macrophages polarize to the classically activated (M1-like) state to perform pro-inflammatory and microbicidal functions. By contrast, cytokines such as IL-4 drive macrophages into the alternatively activated (M2-like) state to perform functions related to tissue repair and helminth clearance[110]. The initial evidence of creatine kinase activity in macrophages came from studies in macrophages from rabbit aveola[111]. During phagocytosis, ATP levels were stable, while the phosphocreatine concentration (normally maintained in threefold to fivefold molar excess over ATP in these cells) decreased by half, suggesting that the creatine kinase–phosphocreatine circuit regulates temporal buffering of ATP in these cells[112,113].

Creatine has been linked to macrophage polarization in support of the M2-like state whilst simultaneously suppressing the classic M1-like state[114] (FIG. 3b). Knockout of *Slc6a8* in macrophages blocked creatine uptake and depleted creatine abundance[114], suggesting that sequestration from the environment is the major route regulating creatine levels in these cells. Specifically, creatine supplementation suppressed the IFNγ–JAK–STAT1–iNOS axis and promoted the IL-4–STAT6–ARG1 axis, which are hallmark molecules of M1-like and M2-like macrophage activation, respectively. The converse was true in *Slc6a8*-knockout macrophages, indicating that creatine controls cellular responses to cytokines such as IFNγ and IL-4 to simultaneously suppress M1-like polarization and activate M2-like polarization.

In addition, creatine influx was enhanced in M2-like macrophages compared with M1-like macrophages. This finding suggests that creatine might be involved in a feedforward cycle where it shifts the balance of macrophages towards an M2-like state, which then in turn have an increased capacity to support the creatine influx that might be required to maintain the M2-like state. *Slc6a8* inactivation in the myeloid lineage (driven by Lyz2-cre) also resulted in considerably decreased M2-like effector function in vivo, while treatment of wild-type mice with exogenous creatine had the opposite effect[114]. Furthermore, mice with Lyz2-cre-driven *Slc6a8* inactivation displayed more severe liver fibrosis than control animals, suggesting that the resolution of liver fibrosis is dependent on creatine levels in macrophages. Intriguingly, creatine and cyclocreatine inhibited cytokine signalling to a similar extent despite having opposite effects on ATP levels. These results strongly suggest that the inhibitory effects of creatine on the IFNγ

pathway occur independently of its classic functions in energy metabolism. Mechanistically, the regulation of macrophage polarization by creatine seems to partly lie in the ability of this metabolite to modulate chromatin accessibility to support a STAT6-mediated transcriptional programme[114]. Creatine could regulate chromatin remodelling indirectly through effects on energy buffering. However, confirmation that modulation of chromatin accessibility occurs through a direct mechanism would be a fascinating finding, and this theory warrants further mechanistic studies.

Macrophages are very active in the uptake of extracellular particles (phagocytosis). This process gives rise to internal compartments called phagosomes. Phagocytosis requires a rapid and spatially restricted reorganization of the actin cytoskeleton, which involves actin polymerization and actomyosin force generation. These molecular processes generate a localized high demand for cellular ATP[115]. A substantial portion of CKB transiently accumulates at nascent phagosome structures to establish a firm functional and spatial connection with the actin-based cytoskeletal machinery to support fast ATP regeneration through its activity in the reverse direction (ATP synthesis)[116]. Thus, by maintaining high local ATP:ADP ratios, CKB supports the phagocytic function of macrophages[116].

Antitumour T cell immunity. T cells have a key role in coordinating anticancer defences[117] and they compete with rapidly proliferating tumour cells for metabolic nutrients. Creatine has been linked to antitumour T cell immunity[118]; tumour-infiltrating T cells increased their expression of *Slc6a8*. In addition to their low expression levels of proteins involved in creatine synthesis (GATM and GAMT), $CD8^+$ T cells probably rely primarily on the uptake of creatine from the extracellular environment. Dietary creatine supplementation suppressed tumour growth in a B16 melanoma model and a MC38 colon cancer model, and creatine supplementation synergized with checkpoint blockade therapies (PD1 and/or PDL1 blockade) to mitigate tumour growth[118]. Thus, creatine seems to be a critical metabolite that supports the maintenance of a high-energy reservoir for cancer-targeting $CD8^+$ T cells.

Immunity: future perspectives. The role of creatine in modulating the function of distinct immune cells under different physiological and pathophysiological conditions will be a ripe area of future research. Creatine clearly has a key role in immunometabolism. A mechanistic understanding of creatine biology in immunity might be used to develop therapies aimed at supporting the metabolic requirements of T cells for combating tumour growth. The intracellular synthesis of creatine also regulates intestinal inflammation. A forward genetic screen identified *Gatm* deficiency as a causative factor in environmentally induced colitis[119]. The importance of creatine in supporting intestinal homeostasis and combating diseases such as inflammatory bowel disease will be interesting future avenues to pursue. Creatine energetics requires energy input, which will come from nutrients available to cells in their local microenvironment. It is conceivable that targeting creatine metabolism might favour nutrient utilization in tumour-resident immune cells compared with cancer cells under specific contexts. Finally, energy buffering seems to be a key function of creatine in immunity. However, exploration of the role of this metabolite in immune function is revealing novel energy buffering-independent pathways controlled by creatine, such as the regulation of transcriptional programmes through effects on chromatin accessibility[114].

Creatine in cancer

Metabolic rewiring in cancer. The development of metastatic cancer cells accounts for 90% of cancer-associated deaths[120]. The reprogramming of cellular metabolism is a hallmark of cancer initiation and progression. Cancer cells can consume or repurpose cellular metabolites in an atypical manner to sustain their rapid proliferation through mechanisms distinct from those used by normal cells. Metabolic adaptations can be exploited to sensitize cancer cells (in both primary tumours and metastatic sites) to existing anticancer drugs. Such findings provide a molecular basis to develop novel therapeutic approaches that efficiently target cancer progression.

Rapidly proliferating tumours outstrip their vascular supply, which induces energetic stress. Cancer cells must adapt their metabolism to these changes in nutrient and oxygen availability by coordinating ATP-producing processes (such as glycolysis and oxidative phosphorylation) and ATP-consuming processes (such as protein synthesis)[121]. In this way, cancer cells maintain energy and redox homeostasis to fuel neoplastic growth under constant energy stress. Normally, glucose in the intracellular space is metabolized to pyruvate, which can then enter the mitochondria to generate reducing equivalents (such as NADH and $FADH_2$) that ultimately generate a proton-motive force across the mitochondrial inner membrane. The energy stored in the proton-motive force is used to drive the synthesis of ATP. However, many cancer cells convert most of their pyruvate to lactate even when adequate oxygen is available — a process called aerobic glycolysis. The current understanding is that cancer cells preferentially use glycolysis because it can feed the anabolic pathways required to produce biomass such as nucleotide, lipid and amino acid synthesis. However, most of the work in this area has focused on cells cultured in vitro and supplemented with supraphysiological levels of available nutrients. In vivo, it is likely that specific nutrients become limiting under certain physiological contexts. Thus, under these nutrient-limited conditions, cancer cells will use the nutrients available to them within their local microenvironment to sustain their survival. For example, activation of a glycolytic molecular programme drives the metastatic colonization and spread of breast cancer cells to the liver[122].

Energy buffering-independent role of creatine in tumour progression. Nutrient utilization by cancer cells will depend on their local microenvironment. Creatine, along with its metabolic derivatives, forms an important system for efficient energy buffering during times of high ATP demand. Phosphorylation of

creatine by creatine kinase generates phosphocreatine. Phosphocreatine can be used in the reverse direction to phosphorylate ADP to ATP, which smooths transient increases in energy consumption by buffering the ATP pool. Some evidence suggests that phosphocreatine has a similar role in malignancy[103,123,124].

However, ample evidence also suggests that this is not always the case. A role for creatine kinase and its substrates in cancer growth was initially demonstrated using the creatine analogue cyclocreatine, which is phosphorylated by creatine kinase. Cyclocreatine was first shown to have antitumour activity in vitro and later in transplanted tumours in vivo[125,126]. This antitumour effect was initially explained by energy depletion due to the trapping of high-energy phosphates via the creatine kinase reaction into phosphocyclocreatine, which is a poor substrate in the creatine kinase reverse reaction[127]. However, because creatine and cyclocreatine both display similar antitumour activity under certain conditions[126,128], a mechanism based purely on cellular energetics can probably be ruled out. For example, cyclocreatine inhibited the growth of colon adenocarcinoma tumours in mice without any indication of energy deficiency[128]. Consistent with this finding, creatine itself inhibited the progression of mammary tumours, neuroblastoma tumours and sarcoma in rats and mice, although higher doses were required than with cyclocreatine in vivo[126]. These data indicate that creatine might have effects on tumour growth that are entirely independent of its role in supporting cellular energy charge. At the same time (noted in a subsequent section) certain cancer cells seem to leverage creatine to support energy metabolism to fuel their growth[103]. We would like to note that the data from the aforementioned studies can also be interpreted as the capacity for cyclocreatine and creatine to indirectly inhibit tumour progression in vivo by their disruption of energy metabolism in tissues that engage in crosstalk with the tumour or its microenvironment.

Creatine kinase expression is induced in many cancers. A common theme regarding the relationship between cancer and creatine is the association between creatine kinase expression and poor prognosis. For example, high levels of CKMT1 are associated with poor prognosis in patients with breast cancer[129] and CKB expression is also elevated in a variety of cancers[130–132]. Importantly, cancer cells that express high levels of CKB were sensitive to the proliferative impairment imposed by cyclocreatine, while cells that expressed low levels of creatine kinase were resistant to the effects of cyclocreatine[125]. Finally, cells with low creatine kinase levels could be sensitized to cyclocreatine by inducing the overexpression of CKB[125].

Liver-metastatic colorectal cancer cells secrete CKB, which synergizes with hepatocyte-derived creatine to drive the formation of phosphocreatine in the extracellular space (FIG. 3c). The influx of phosphocreatine into colorectal cancer cells was used to fuel metastatic survival, possibly through intracellular production of ATP[103]. Thus, crosstalk between hepatocytes and metastatic liver cancer cells would require both local creatine synthesis within hepatocytes and an influx of creatine into cancer cells through CRT. Phosphocreatine supplementation has been reported to rescue the depleted ATP levels in cancer cells, suggesting that colorectal cancer cells atypically import phosphocreatine through CRT[103].

Additional work has demonstrated that HER2$^+$ breast cancer cells induce a signalling cascade that converges on the phosphorylation of CKMT1 on tyrosine 153 (Y153) in a tyrosine-protein kinase ABL1-dependent manner[124]. Y153 phosphorylation of CKMT1 stabilized the protein to support phosphocreatine shuttling and to drive proliferation. Genetic depletion of CKMT1 or pharmacological inhibition of phosphocreatine metabolism with the creatine analogue cyclocreatine decreased phosphocreatine levels and impaired proliferative capacity in cultured cells and xenografts. The proliferative defects in cyclocreatine-treated cells were rescued by the supplementation of media with phosphocreatine. Combination therapy of cyclocreatine with the HER2 kinase inhibitor lapatinib reduced the growth of xenografts from patients with HER2$^+$ breast cancer. Importantly, despite the HER2-directed monoclonal antibody trastuzumab having substantial activity against HER2$^+$ breast cancer cells, these cells can acquire resistance to this drug. Cyclocreatine inhibited phosphocreatine shuttling and exhibited antitumour activity against HER2$^+$ breast cancer cells that were resistant or sensitive to trastuzumab. Collectively, these data suggest that targeting phosphocreatine metabolism can enhance the efficacy of existing breast cancer therapies.

The proto-oncogene *MECOM* (also known as *EVI1*) is the site of chromosomal translocations in some cases of acute myeloid leukaemia and these events are associated with poor clinical outcomes[133]. CKMT1 is necessary for survival of MECOM-expressing cells in patients with acute myeloid leukaemia and has also been linked to an oncogenic transcriptional programme in MECOM$^+$ acute myeloid leukaemia[123]. Mechanistically, MECOM activated CKMT1 expression by inhibiting the myeloid differentiation regulator RUNX1. Depletion of CKMT1 or use of cyclocreatine promoted cell cycle arrest and apoptosis of MECOM$^+$ cells and prolonged the survival of orthotopic and genetic models of acute myeloid leukaemia. The inhibition of creatine metabolism with cyclocreatine was associated with the impaired expression of genes associated with GSK3 and Wnt signalling[134]. Again, phosphocreatine could rescue ATP levels and proliferative capacity in cyclocreatine-treated and CKMT1-depleted cells[123]. This study explored some aspects of this mechanism and noted that phosphocreatine supplementation could induce creatine biosynthesis intracellularly as labelled arginine was traced into guanidinoacetate and creatine. Thus, phosphocreatine might not be imported into the cell, but somehow promotes intracellular creatine synthesis. However, how this activity replenishes the ATP pool is still unknown.

Creatine in cancer: future perspectives. It is currently unknown whether the rescue of ATP levels by the addition of exogenous phosphocreatine occurs directly by the buffering of ATP through phosphocreatine transport into cells and by the phosphotransfer activity of cytosolic creatine kinase in the reverse (ATP-generating) direction. Further investigation is needed to determine

the mechanism by which CRT can transport both creatine and phosphocreatine, two molecules with distinct charges. Notably, current data do not rule out an indirect effect of extracellular phosphocreatine on intracellular levels of ATP. For example, phosphocreatine is known to inhibit the cell surface enzymes of AMP catabolism, AMP deaminase and 5′-nucleotidase[135]. This action would indirectly spare AMP levels, which could then be converted to ADP intracellularly through adenylate kinase (AMP plus ATP gives two ADP). ADP could subsequently be used to generate ATP through glycolysis or oxidative phosphorylation. AMP deaminase is also upregulated in several cancers[136,137] and so could potentially be therapeutically targeted.

Interestingly, phosphocreatine and phosphocyclocreatine exhibited low-affinity interactions with phospholipids[138]. These interactions change the phospholipid bilayer properties and protect lipid membranes against permeabilization and cell lysis. These data indicate that creatine has a role in membrane biology that is independent of its effects on energy buffering. This action of phosphocreatine with membrane phospholipids can stabilize membranes, possibly affecting ion homeostasis and cell signalling. Exogenous phosphocreatine also inhibits lipid peroxidation mediated by reactive oxygen species[139]. This area of research certainly merits further investigation, particularly in cancer biology, where creatine is critical for cancer cell survival.

The release of creatine kinase into the circulation has long been known to occur in patients with myocardial infarction, muscle trauma, alcohol use disorder, some endocrine disorders, certain cancers and malignant hyperthermia[140]. This non-regulated release from cells (primarily muscle) is understood to occur because of cellular damage. Fairly little is known regarding pathways that trigger creatine kinase secretion in a regulated manner from cells. Furthermore, CKB has been reported to be secreted from colorectal cancer cells[141], even though it does not have a classic N-terminal signal peptide. The molecular details of unconventional creatine kinase secretion remain to be determined.

The strong epidemiological associations between adipose tissue mass and the incidence (and mortality) of a variety of malignancies, including breast, renal, colon, oesophageal and pancreatic cancer, strongly suggest that obesity is a major modifiable risk factor for cancer[142–144]. The molecular basis for the association between obesity and certain cancers is not well understood but is probably multifactorial[142,145]. Thus, a more complete understanding of the mechanisms linking obesity to cancer is required to develop therapeutic approaches to oppose obesity-driven tumorigenesis. The incidence of liver metastasis is increased in individuals with obesity[146,147], which seems to be at least partly due to changes in hepatocyte metabolism[103,148,149]. Excess lipids not incorporated into hypertrophic adipocytes can spill over into the liver during obesity, resulting in diseases such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH)[150]. Thus, the incidence of liver metastasis can be attributed, in part, to the obesity epidemic and to the subsequent development of NAFLD and NASH[148,149].

In the setting of obesity and NAFLD, the liver increases its creatine biosynthetic capacity through the upregulation of GAMT, the key enzyme in creatine synthesis in hepatocytes[151]. Liver-derived creatine is appropriated from the extracellular milieu to promote liver metastatic growth and support the rapid proliferation of certain metastatic liver cancers such as those from colorectal cancer cells[103]. These data indicate that cancer cells in the liver microenvironment can leverage the use of creatine to fuel survival and metastatic progression. Thus, it seems plausible that elevated creatine synthesis in the liver might be responsible for the increased metastatic burden that occurs in obesity. However, certain gaps in knowledge exist. First, it is unknown if creatine-dependent liver-metastatic colonization is limited to colorectal cancer cells. For example, breast cancer cells can metastasize to the liver[122]; therefore, it remains to be seen if liver-metastatic breast cancer cells can utilize creatine to fuel their survival to the same extent as colorectal cancer cells that metastasized to the liver. Second, how cancer cells that have metastasized to the liver will adapt to a microenvironment that does not contain creatine has not been examined. Presumably, these cells will acquire metabolic alterations that enable them to bypass the colonization impairment that might arise in the context of limited creatine availability. These alternative biochemical pathways might then become metabolic vulnerabilities that can be exploited for therapeutic gain.

Conclusions

Creatine kinase catalyses the reversible transfer of a phosphoryl group from ATP to the guanidinium group of creatine in an equilibrium reaction[6]. Thus, phosphocreatine accumulation, like a battery, serves as a high-energy carrier to regenerate levels of ATP in the reverse creatine kinase reaction to maintain high ATP:ADP ratios during periods of high ATP consumption. Phosphocreatine also diffuses faster than ATP owing to its smaller size. In addition, because creatine kinase isoenzymes are distributed at discrete sites within the cell, phosphocreatine can transmit chemical energy to the required sites of high ATP demand through the creatine kinase–phosphocreatine circuit. Most cell types express at least two creatine kinase isoenzymes (one mitochondrial and one cytosolic)[6,12,15,17]. Through the expression of primarily one creatine kinase isoenzyme, some cells can leverage the capacity of creatine kinase to redistribute to discrete subcellular locations and carry out its canonical reversible phosphoryl transfer activity[17,152]. The regulation of how creatine kinase isoenzymes can traffic to distinct subcellular compartments is a key area to unravel in the field.

CKB buffers ATP levels at sites of membrane ruffling, which is critical for cell spreading and the migration of astrocytes and fibroblasts[153]. Similarly, it has long been known that creatine kinase is critical for supporting the motility of spermatozoa[154,155]. Thus, the classic spatiotemporal buffering function of the creatine kinase–phosphocreatine circuit might influence cytoskeletal dynamics in a diverse range of cell types that move. On a similar note, creatine energetics might support cancer

cell invasion into the tissues surrounding the primary tumour and might play a key role in the extravasation of cancer cells into the metastatic niche[156]. Clearly, understanding the control of creatine energetics might be relevant for understanding disease processes.

The newly identified functions for creatine energetics do not necessarily involve new biochemistry of the creatine kinase reaction. For example, in the mitochondria of thermogenic adipocytes, creatine is understood to release ADP at levels in excess of what would be expected if creatine was placed in a biochemical pathway that supported a 1:1 stoichiometric relationship between creatine and ATP[157]. The excess oxygen consumption with a given amount of creatine in the mitochondria of thermogenic adipocytes suggests that an enzyme exists that has phosphocreatine hydrolytic activity. Thus, soon after phosphocreatine is generated, it is hydrolysed back to creatine. Creatine can then initiate another cycle of ATP turnover (FIG. 3a). In macrophages, creatine might utilize novel biochemistry as its inhibitory role on IFNγ-mediated signalling seems to be independent of its role in energy buffering[114] (FIG. 3b). Moreover, the connection between creatine and chromatin accessibility needs to be further investigated to determine whether creatine also modulates chromatin remodelling in an energy-independent manner. Finally, in some cancer cells, CKB seems to be secreted in a regulated manner to perform typical creatine kinase biochemistry, albeit extracellularly (FIG. 3c). Atypical transport of phosphocreatine by CRT suggests that this transporter might have unique properties that enable it to recognize and transfer phosphorylated and non-phosphorylated forms of creatine. Of course, these new proposed functions for creatine in these diverse cell types are not mutually exclusive with the well-established roles of creatine in spatiotemporal energy buffering. This classic function of creatine most likely also has a role in thermogenic adipocytes, immune cells and cancer cells.

Energy buffering by the creatine kinase–phosphocreatine circuit might be particularly important for cells where the mitochondrial network cannot transduce energy through the reticular network. The creatine kinase–phosphocreatine circuit has been proposed to have evolved to meet the energy demands of highly polarized cells such as spermatozoa or choanocytes (the cells that generate water currents in sponges[157]). For example, neurons extend their axons and dendrites for millimetres and centimetres and, in the case of human peripheral nerves or corticospinal tracts, up to a metre. This feature contrasts with most other cells, which are measured in micrometres[158]. Thus, the neuron poses an extreme case for mitochondrial distribution and the need to supply energy to far-flung cellular regions. Indeed, neuronal regions with the highest ATP demand are the synapses, which are located at the extremities of the cell far from the majority of the ATP-generating mitochondria[158]. Similarly, vertebrate photoreceptor cells of the retina and spermatozoa exhibit a polarized organization. ATP-utilizing reactions of these cells are spatially distinct from the oxidative phosphorylation and mitochondrial creatine kinase that supply ATP and transphosphorylate it to phosphocreatine, respectively[159].

Furthermore, mitochondria in brown adipocytes undergo fission during thermogenic respiration[85], which might necessitate phosphocreatine-mediated energy shuttling to support high local levels of ATP to maintain proper adipocyte function. Indeed, disruption of the mitochondrial network upon thermogenic activation might be necessary to limit the propagation of leak-mediated proton-motive force dissipation by UCP1-expressing organelles. By contrast, mitochondria have been proposed to represent a united electrical system that could facilitate energy delivery from the cell periphery to the cell core[160]. For example, the extensive mitochondrial network in skeletal muscle is sufficient to transduce energy over large distances because diffusion distances of metabolites are minimized[9]. This finding indicates a limited reliance on spatial energy buffering by the creatine kinase–phosphocreatine circuit and a greater reliance on energy distribution through the conductance of mitochondrial membrane potential. This feature might partly account for the mild skeletal and cardiac muscle phenotypes of global creatine kinase knockout mouse models[161–165]. For example, *Ckm*$^{-/-}$/*Ckmt2*$^{-/-}$ mice exhibit no overt effects on heart rate, locomotor activity or behaviour, suggesting that the absence of creatine kinase activity in muscle does not pose an obstacle to normal heart and skeletal muscle function[161].

The relative ease of phosphocreatine diffusion is an important factor in supporting high ATP:ADP ratios at distant sites with high ATP consumption. In addition, creatine kinase isoenzymes might distribute to discrete locations to support the long-range intracellular transport of mitochondria, which is primarily accomplished by microtubule-based motors (kinesins and dynein)[166]. These architectural aspects are not neuronal specific; mitochondria redistribute along actin and tubulin networks in migrating lymphocytes and tumour cells[167,168]. These are the precise scenarios where the creatine kinase–phosphocreatine circuit would be predicted to have a key role. Obviously, organelle architecture is not static. All cells probably modify their mitochondrial dynamics depending on their environment. In sum, these data suggest an association between creatine biology and mitochondrial dynamics. However, whether alteration of the mitochondrial architecture can lead to changes in the requirement for energy buffering by the creatine kinase–phosphocreatine circuit awaits investigation.

There is a clear association between overweight and obesity and inflammation. Macrophages represent up to 10% of cells in healthy adipose tissue, and can increase to 50% within the obese adipose tissue microenvironment, taking on a pro-inflammatory phenotype[169–171]. Obesity causes ectopic lipid deposition in non-adipose tissues[172], including cells of the immune system[173]. These changes disrupt tissue integrity and lead to alterations in lymphoid tissues, thereby changing the cellular milieu[173]. Compared with white adipose tissue, BAT is less susceptible to developing local inflammation in response to obesity. However, defective BAT can induce a high pro-inflammatory environment, such as with *Ucp1* deletion[34]. It will be intriguing to understand whether

impaired thermogenic adipose tissue enhances systemic inflammation, which could substantially influence host metabolism and nutrient utilization. The capacity for creatine to influence this relationship by acting on immune cells or adipocytes will be an interesting area to pursue in the future.

The study of creatine in cell types (that is, adipocytes, immune cells and cancer cells) where this metabolite has historically not been generally appreciated has sparked many of the latest advances in the field. These investigations have begun to reveal the plethora of functions that creatine has in cellular and systemic energy metabolism. Notably, most alleles of the creatine pathway (synthesis, transport and phosphoryl dynamics) have not been evaluated in a tissue-specific context in vivo owing to a lack of genetic models. The development of these genetic tools will be critical to progress the field of creatine biology.

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