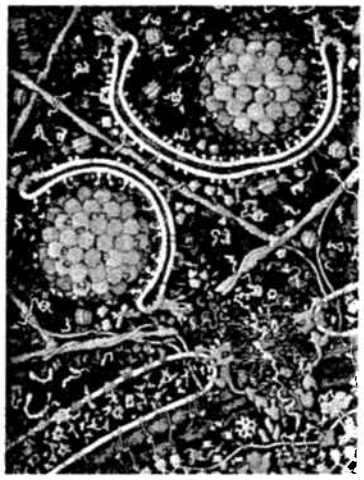

INTRODUCTION

Metabolism Is Not Boring

SO INVESTIGATOR STEVEN MCKNIGHT ADMONISHED HIS AUDIENCE LAST SPRING after a presentation at a conference (on metabolism and cancer, no less). He may have been "preaching to the choir" in this case, but what led him to issue such a blunt reminder? It seems that for a generation of biological scientists, metabolism was an area of biochemistry to be mastered and then put aside. Metabolism was always there in the background, providing the cell with the energy and resources to do what was required, but was rarely recognized to determinedly influence, and be influenced by, the physiological state of the cell.

This special issue of *Science* celebrates a resurgence of interest in metabolism and an appreciation of its central role in disparate areas of cell biology, physiology, medicine, and synthetic biology (www.sciencemag.org/special/metabolism/). McKnight's Perspective (p. 1338) takes a broad look at the field and justifies his excitement that recognizing the reciprocal regulation of metabolism and other cellular processes promises to advance our understanding of complex physiology.

There is renewed interest in the metabolism of cancer cells and its potential as a therapeutic target. Levine and Puzio-Kuter (p. 1340) review metabolic changes in cancer cells, as well as the recent suggestion that alterations in a metabolic enzyme can lead to the production of an "oncometabolite" that supports cancer cell growth. When energy sources are limited, cells use a process known as autophagy for breaking down cellular components to provide substrates for metabolism. Rabinowitz and White's Review (p. 1344) discusses roles of autophagy in metabolism, its regulation, and its implications for cancer and degenerative diseases.

Of course not all fields have neglected metabolism over the years, and an enormous literature describes the role of insulin and related hormones in controlling cellular metabolism. In a Focus Issue of *Science Signaling*, a Research Article and Perspective describe an unexpected signal from the insulin receptor that confers sensitivity to cell death when insulin is not present. Also highlighted are a role for lipids as cellular sensors of glucose metabolism and a mechanism by which cells can survive oxidative stress by shifting the activity of the cell's protein degradation complex, the proteasome.

In *Science Translational Medicine*, Vallerie and Hotamisligil review how in a strategy to combat obesity and insulin resistance, a therapeutic inhibitor of cell signaling pathways must have coordinated actions in multiple cell types. Back in *Science*, Bass and Takahashi (p. 1349) review new insights into the interaction of metabolism with circadian clocks. Displacement of eating and periods of activity away from the normal light-dark cycle, as experienced in jet lag or shift work, have marked effects on metabolic diseases.

Cellular metabolic pathways, particularly in yeast or bacteria, can be exploited to synthesize compounds that are difficult or expensive to produce by other means. Keasling (p. 1355) reviews advances in metabolic engineering and looks forward to a future in which customized microbes made by computer-aided design can efficiently produce desired chemicals, ranging from fuels to pharmaceuticals.

See? It's not boring at all! – L. BRYAN RAY

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Metabolism

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See also Science Translational Medicine *and* Science Signaling *at www.sciencemag.org/special/metabolism/.*

Science

PERSPECTIVE

On Getting There from Here

Steven L. McKnight

Studies on a variety of interesting biological problems, ranging from circadian rhythm to cancer cell growth to longevity, have begun to give evidence that the physiological state of cells and tissues reflects both the cell's regulatory systems and its state of intermediary metabolism. It is appreciated that the regulatory state of a cell or tissue, as driven by transcription factors and signaling pathways, can impose itself upon the dynamics of metabolic state. It follows that the reciprocal must also be the case, that metabolic state will feed back to impose itself on regulatory state. An appreciation and understanding of this reciprocity may be required to crack open problems in biological research that have heretofore been insoluble.

For the past 30 years, research in the biological sciences has been dominated by molecular biology. The successes of this approach have shaped our understanding of innumerable domains of biology. But any field that becomes sufficiently muscular can overshadow other credible approaches to scientific inquiry. One field etiolated by the cloud of molecular biology has been metabolism (Fig. 1). The vast majority of discoveries made by molecular biologists over the past several decades required no attention to the metabolic state of a cell. Molecular biologists needed no distracting thoughts about the metabolic state of a cell to discover microRNAs, the reprogramming of somatic cells into pluripotent stem cells, or gene rearrangement as the underlying basis for the generation of antibody diversity.

One simple way of looking at things is to consider that 9 questions out of 10 could be solved without thinking about metabolism at all, but the 10th question is simply intractable. As the saying goes, you simply "can't get there from here" to answer this 10th question if you are ignorant about the dynamics of metabolism. This, I propose, is where we are now finding pregnant opportunities in the field of experimental biology. The low-lying fruit that could be picked by molecular biologists without having to consider the metabolic state of a cell, tissue, or organism is largely gone. The more sticky problems that required attention to the dynamics of metabolism and that were pushed aside for decades now loom as interesting and important challenges.

Consider a prime example of how molecular biologists have begun to embrace the importance of metabolic regulation. Cancer researchers have long known of the enigmatic ability of tumor cells to undertake aerobic glycolysis, the so-called Warburg effect (*1*, *2*). It makes sense that cancer cells would be highly glycolytic, yet why would these cells choose to dispose of the terminal product of glycolysis? Instead of allowing pyruvate to be converted into acetyl–coenzyme A (CoA) via the spectacularly beautiful pyruvate dehydrogenase enzyme complex within mitochondria, cancer cells instead convert pyruvate into lactate through the lactate dehydrogenase enzyme, and then simply excrete it—blindly giving away exceptional energetic value stored in the lactate hydrocarbon. Any cell that wants to grow—and there is nothing cancer cells care more about than growth—would be crazy to waste hydrocarbon; this would be akin to a motorist driving down the New Jersey Turnpike throwing away gasoline. The spendthrift waste of lactate likewise deprives the cancer cell of huge amounts of acetyl-CoA to be used for the synthesis of lipids, sterols, and other cellular building blocks. Despite progress, attention, and plenty of hype, it is safe to conclude that the famous Warburg effect remains a mystery.

Cancer researchers now recognize that regulatory proteins, such as the hypoxia-inducible transcription factors, can directly regulate the expression of genes encoding glycolytic enzymes (*3*). They now pay attention to how their favorite regulatory proteins, including the Myc and p53 transcription factors, help set the metabolic state of cells. The fact that these masterful transcription factors participate in dictating the metabolic state of a cell is now beginning to be accepted. The equally compelling corollary, however, remains largely unappreciated. That is, if regulatory state

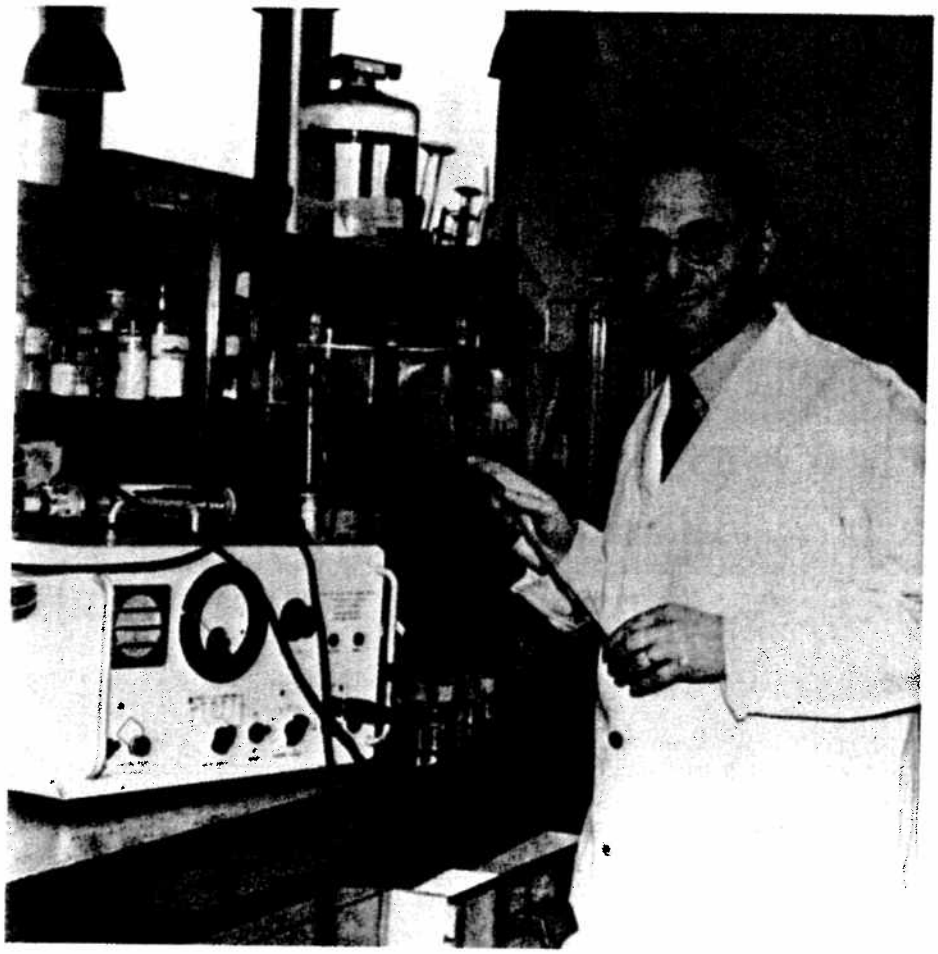

Fig. 1. All eyes were focused on metabolism back in 1953 when Hans Krebs received the Nobel Prize for his discovery of the citric acid cycle.

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(transcription factors, signaling pathways, etc.) is accepted to control metabolic state, is it not also unconditionally certain that metabolic state will reciprocally control the regulatory state itself? Understanding this reciprocity, and digging to the bottom of it, is where the future lies. Perhaps fittingly, this research will require the sophistication of scientists having genuine skills in the study of enzymology and intermediary metabolism.

Whole-genome sequencing efforts of individual tumors, now numbering in the thousands—yet soon to be numbered in the hundreds of thousands—are providing unbiased views of the myriad of "oncogenotypes" that underlie human cancer. Instead of ignoring mutations that happen to fall in the genes encoding metabolic enzymes, scientists seem more keen than ever to find and study such mutations. This change may reflect the recognition by cancer researchers that mutations that alter the function of metabolic enzymes might help to resolve the enigmatic, aerobic glycolytic state of certain cancer cells. It is equally likely that an understanding of how cancer cells veer away from normality with respect to intermediary metabolism might lead to the conceptualization of new and inventive strategies for therapeutic intervention.

For example, a set of recurrent genetic lesions believed to influence the metabolic state of glioblastoma cancer cells have been identified in the genes encoding either of the two isoforms of isocitrate dehydrogenase (*4*, *5*). Perplexingly, most, if not all, of these lesions mutate a single arginine residue in either of the two isoforms of the enzyme (IDH1 or IDH2). The precise selectivity of the mutational events, coupled with the observation that only one allele of either enzyme appears to be mutated in human cancer, pointed to the possibility that the lesions might be causing the enzymes to adopt a new catalytic function. Indeed, the mutated forms of the IDH1 and IDH2 enzymes exhibit a reduced affinity for isocitrate and are endowed with a new catalytic function wherein α-ketoglutarate is converted in an NADPH (nicotinamide adenine dinucleotide phosphate, reduced)–dependent manner to 2-hydroxyglutarate (*6*).

What pathways might be expected to be altered in a cell impeded for the production of α-ketoglutarate and concomitantly endowed with increased intracellular production of 2-hydroxyglutarate? A logical guess would be the family of dioxygenase enzymes that use α-ketoglutarate as an essential cofactor and, simultaneously, are inhibited in the presence of 2-hydroxyglutarate. Included among this family of dioxygenase enzymes are the prolyl-hydroxylase enzymes that modify and negatively regulate the HIF-1α (hypoxia-inducible factor 1α) and HIF-2α transcription factors in oxygenated cells (*7*, *8*). Partial elimination of these dioxygenase enzymes could be interpreted to lead to the activation of the hypoxia response pathway, thereby accounting for the activation of transcription of genes encoding glycolytic enzymes just as happens in the absence of the Von Hippel–Lindau (VHL) tumor suppressor gene (*9*, *10*). This interpretation is very likely oversimplistic. Attenuation of α-ketoglutarate concentrations and accumulation of increased amounts of 2-hydroxyglutarate almost certainly lead to the inhibition of other dioxygenase enzymes, of which mammalian cells have scores of isoforms. Intriguingly, some of these additional dioxygenase enzymes have been implicated in the control of histone demethylation (*11*–*13*), leaving open the possibility that changes in metabolic state might impose alterations in the epigenetic state of cancer cells.

The drift of this thinking is concordant with the recent discovery of mutations found in renal cell cancers in the mitochondrial enzyme fumarate hydratase that converts fumarate to malate (*14*). These mutations are more rare and of a recessive nature—wherein both alleles of the gene encoding fumarate hydratase must be inactivated. Because fumarate is known to be an inhibitor of the aforementioned family of dioxygenase enzymes, it is conceptually logical to hypothesize that the accumulation of excessive amounts of fumarate might inactivate the prolyl-hydroxylase enzymes that normally keep the HIF transcription factors in an inactive state (and, perhaps, likewise impose alterations in the epigenetic state of cancer cells). Finally, the same reasoning might apply to rare mutations in the human genes encoding mitochondrial succinate dehydrogenase and its assembly factors that are believed to contribute to the formation of paragangliomas and pheochromocytomas (*15*, *16*). The exciting angle on these studies of cancer-causing mutations in the genes encoding isocitrate dehydrogenase, fumarate hydratase, and succinate dehydrogenase is that they formally predict the concept that the metabolic state of a cell can indeed exert control over its regulatory state, thereby confirming the reciprocal relationship between the two.

One enduring complication that shows little sign of resolution concerns the manner in which cancer cells are grown and studied in tissue culture plates. We routinely grow cancer cells under conditions of unlimited access to glucose—not to mention oxygen, vitamins, known and unknown growth factors present in serum, and every nutritional fertilizer imaginable. The growth environment of cancer cells within tumors, especially solid tumors, could hardly be more different than that of cells being grown under standard, tissue culture conditions. It is reasonable to suspect that cancer cells weave their way through exceptionally selective oncogenetic gymnastics to achieve a growth-permissive metabolic state. If so, what features of this metabolic state can be anticipated to be preserved and studied when cells are practically grown in Karo syrup or alongside floating logs of Snickers Bar candy? Returning to the "you can't get there from here" theme, it is predictable that we will have to find more biologically sound ways to grow cancer cells in culture in order to favorably use them in efforts to discover therapeutics that might exploit their unique metabolic state.

The resurrection of research involving or including metabolism is clearly upon us—that is the good news. The bad news is that the field was sufficiently snuffed over the past several decades that we have precious few scientists who have been trained to genuinely understand intermediary metabolism. Just because we can now pronounce the names of the metabolic enzymes whose encoding genes and mRNAs show up on our ChIP-Seq (chromatin immunoprecipitation–sequencing) and DNA microarrays lists does not necessarily mean that we can put two and two together. Despite the handicap of not being able to field an experienced team at this point, it is encouraging to see favorable trends that may enable negotiation of discovery routes that were, until now, largely obscure.

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17. I thank M. Brown, R. Bruick, B. Tu, and J. Rutter for helpful editorial comments.

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REVIEW

The Control of the Metabolic Switch in Cancers by Oncogenes and Tumor Suppressor Genes

Arnold J. Levine[1,2]* and Anna M. Puzio-Kuter[2]

Cells from some tumors use an altered metabolic pattern compared with that of normal differentiated adult cells in the body. Tumor cells take up much more glucose and mainly process it through aerobic glycolysis, producing large quantities of secreted lactate with a lower use of oxidative phosphorylation that would generate more adenosine triphosphate (ATP), water, and carbon dioxide. This is the Warburg effect, which provides substrates for cell growth and division and free energy (ATP) from enhanced glucose use. This metabolic switch places the emphasis on producing intermediates for cell growth and division, and it is regulated by both oncogenes and tumor suppressor genes in a number of key cancer-producing pathways. Blocking these metabolic pathways or restoring these altered pathways could lead to a new approach in cancer treatments.

In 1926, Otto Warburg demonstrated that cancer cells did not metabolize glucose in the same way that glucose was catabolized in normal, adult differentiated cells (*1*, *2*). The cancer cells relied on glycolysis, even in the presence of abundant oxygen (aerobic glycolysis) with a reduced use of the tricarboxylic acid (TCA) cycle, so that the pyruvate made in glycolysis was commonly converted to lactate, which was secreted from the cell (Fig. 1). Warburg suggested that this observation explained the cancer phenotype and was possibly a causal event in cancer formation (*1*, *2*). There were several reasons why these observations were not understood and did not promote additional research. First, metabolizing glucose by glycolysis to produce pyruvate and secreted lactate is energetically inefficient. Most of the adenosine triphosphate (ATP) generated by glucose catabolism (34 out of 36 ATP molecules per molecule of glucose) occurs during the TCA cycle, which is used less often in cancer cells (Fig. 1). Second, it was unclear how this observation could contribute to the cancer phenotype. Third, possible mechanisms to mediate a switch to glucose utilization in glycolysis from the more efficient movement of pyruvate into the mitochondria to produce acetyl–coenzyme A (CoA) and enter the TCA cycle were only poorly understood. Fourth, with the discovery of the mutational activation of oncogenes and inactivation of tumor suppressor genes as causal steps in cancer, the relationship between these mutant genes and metabolic regulation was unclear. Remarkably, after a long absence of interest, research done in the past 10 years has begun to answer these questions. Although our understanding of each question is still imperfect, it is becoming clear that both oncogenes and tumor suppressor gene products can influence the switch between aerobic glycolysis and a more extensive use of the TCA cycle to generate ATP. Furthermore, the altered metabolic processing of glucose observed by Warburg may well contribute to some of the causal changes in the cancer phenotype. There have been a number of reviews that emphasize different aspects of this question and provide a diverse set of answers (*3*–*9*).

Normal cells and cancer cells use both glucose and glutamine as substrates to generate energy for the cell (ATP); to produce substrates to synthesize amino acids, nucleosides, and fatty acids; and to regulate the redox potential (number of oxidized molecules in a compartment divided by number of reduced molecules) so as to minimize the effects of reactive oxygen species (ROS) that damage membranes and proteins and cause mutations in a cell. Glucose contributes carbon, oxygen, and hydrogen for both anabolic processes and energy, whereas glutamine contributes nitrogen for synthesis of purines, pyrimidines, and nonessential amino acids. Metabolism of glutamine also produces the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) for the synthesis of fatty acids and the modulation of the redox potential in a cell. Glucose passing through the pentose phosphate pathway (PPP) also generates NADPH and ribose-5-phosphate for the synthesis of nucleotides (Fig. 1). Normal adult differentiated cells have a low cell division rate (low turnover) and predominantly metabolize glucose to CO_2 and H_2O through glycolysis and the TCA cycle. This satisfies the needs of these cells for free energy supplied by efficient ATP generation during oxidative phosphorylation (complexes 1 to 4 in the oxidative phosphorylation chain) linked to the TCA cycle in mitochondria. There are several times, however, when regulated rapid cell division is required, such as during embryonic development, in wound healing (liver regeneration), or in the immune responses to specific antigens, where clonal selection provides increased cell numbers with increased immune specificity. Cancer cells share many of these same requirements for energy, substrates to grow and divide, and control of the redox potential and ROS in the cell.

What these processes have in common is a need to synthesize substrates for membranes, nucleic acids, and proteins (increase mass), which means not metabolizing all of the glucose to CO_2 and H_2O but instead providing the proper intermediates for cell growth. This is accomplished, in part, by slowing the entry of pyruvate into mitochondria, decreasing the conversion to acetyl-CoA, and slowing the rate of the TCA cycle. The pyruvate that builds up in aerobic glycolysis is, in part, converted into lactate that is secreted, eliminating it from the pool and keeping glycolysis active. The secreted lactate lowers the pH of the cellular environment and the extracellular matrix. This may influence remodeling of the matrix, permitting blood vessel invasion in response to angiogenic factors produced by the tumor (*10*). Furthermore, as a consequence of glycolysis, tumor lesions can become acidotic, which allows for the selection of motile cells that can break through the basement membrane and metastasize. The last step in glycolysis is catalyzed by pyruvate kinase, which receives input about both anabolic precursors and the energy status of the cell. Cancer cells make the fetal isoform of pyruvate kinase (the M2 isoform), which is a spliced variant of the gene that adds several amino acids, one of which is a tyrosine. This tyrosine is phosphorylated in cells with activated tyrosine kinase signaling, a hallmark of actively growing cells. Pyruvate kinase M2 is stimulated in a feedforward loop by fructose 1,6-bisphosphate, but the phosphotyrosine inhibits this positive regulation. Thus, in cancer cells the last step of glycolysis is slowed, resulting in a buildup of phosphorylated intermediates that can be used in anabolic synthesis and cell growth (*11*).

Rapidly dividing cells require favorable energetics [that is, higher ATP/adenosine diphosphate (ADP) and ATP/adenosine monophosphate (AMP) ratios]. Many cancer cells satisfy this problem by taking up much larger amounts of glucose than do normal cells. This results from facilitated glucose transport by one or more of several isozymes of membrane glucose transporters (GLUT 1 to 9). Once inside the cell, glucose is phosphorylated by one of several hexokinase enzymes (the first step in glycolysis) to keep it in the cell because of the charge added to glucose (Fig. 1). The high concentrations of glucose in the cells of a cancer may be observed by positron emission tomography (PET) scans of radioactive F-19-2-deoxyglucose (FDG is not metabolized but is located in the

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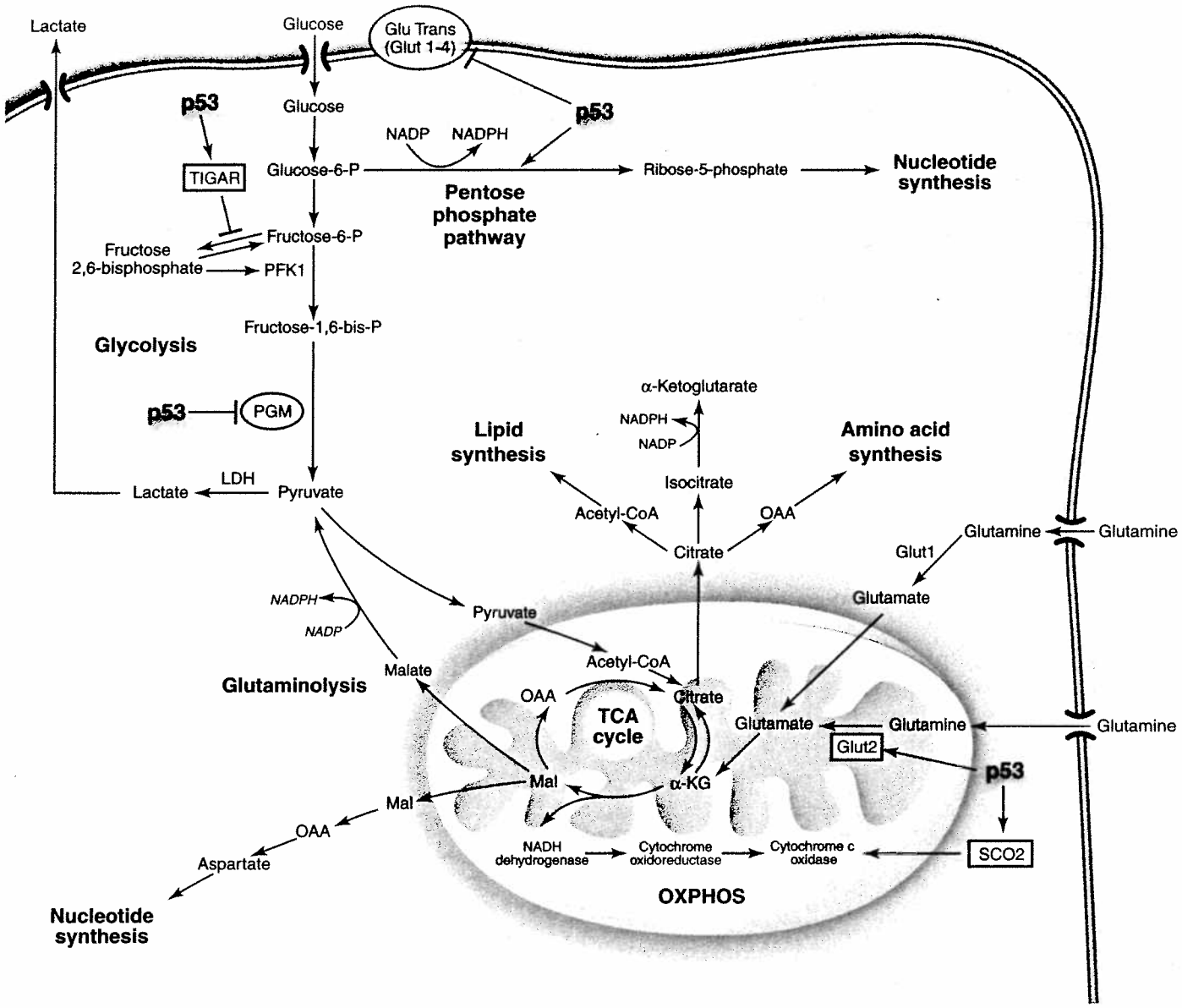

Fig. 1. Signaling networks and their regulation of metabolism in proliferating cells. The figure shows aspects of metabolism in proliferating cells including glycolysis; lactate production; TCA cycle; oxidative phosphorylation; PPP; glutaminolysis; and the biosynthesis of nucleotides, lipids, and amino acids. Glucose can be processed through glycolysis for production of ATP and pyruvate, pass through the PPP to generate ribose 5-phosphate and NADPH, and also enter into the mitochondrion-localized TCA cycle. Glucose-derived citrate is exported to the cytosol and processed to acetyl-CoA, oxaloacetate (OAA), or α-ketoglutarate (α-KG). Glutamine is deaminated to form glutamate, which is processed to generate α-ketoglutarate and maintain the TCA cycle. p53 induction of key players is boxed, and p53 inhibition is circled. p53 induces TIGAR, inhibits phosphoglycerate mutase (PGM), and represses GLUT1 and GLUT 4, resulting in inhibition of glycolysis and opposing the Warburg effect that is seem in many cancers, whereas p53 induction of SCO2 and GLS2 enhances mitochondrial respiration. Glut Trans indicates glucose transporters; Glut 1, glutaminase 1; Glut 2, glutaminase 2; LDH, lactate dehydrogenase; Mal, malate; and OXPHOS, oxidative phosphorylation.

cell), which is indicative of enhanced glucose uptake by cells. Many, but not all, cancers have this property (*3*–*9*) of increasing glucose uptake, and this is a confirmation of the Warburg effect.

With large amounts of glucose available in a cell, glucose is metabolized through the PPP, producing nucleosides and generating NADPH. The NADPH is essential for fatty acid synthesis, along with acetyl-CoA (which is made from some of the pyruvate in mitochondria that is not converted to lactate). NADPH also contributes to a proper redox control and protects the cell from ROS. There are several ways the cell responds to lower ROS levels, but by far the major molecule involved is glutathione (GSH), which eliminates ROS by accepting an electron and is converted to its oxidized form, GSSG (glutathione disulfide). The enzyme glutathione reductase uses NADPH to reduce GSSG to GSH. Thus, NADPH is a major source of cellular "coolant" when oxidative reactions run too "hot" (high ROS levels) by using large amounts of glucose to produce both substrates and energy. However, high levels of ROS can be advantageous for cancer cells when they allow for the stimulation of cell proliferation, induction of genetic instability, and evasion from senescence. Although if levels are too high, then

cancer cells undergo oxidative damage–induced cell death. Thus, ROS levels can be exploited to selectively kill cancer cells and therefore be used as a potential therapeutic.

Glutamine contributes both to substrate needs of a dividing cell and to control of redox potentials through the synthesis of NADPH. As with glucose, excessive amounts of glutamine are taken up (by a glutamine transporter) and used by cancer cells. After glutamine is taken into the cell, a mitochrondrial-associated enzyme, glutaminase-1, converts it to glutamate (a transaminase can use the amino group and capture the nitrogen for synthesis of nucleosides, and amino acids or ammonium is produced). Glutamate is converted to α-ketoglutarate and enters the TCA cycle in the mitochondria. The malate and citrate produced in the TCA cycle leave the mitochondria, where malate is converted to pyruvate plus NADPH and citrate is converted to isocitrate and then to α-ketoglutarate, generating another molecule of NADPH. Citrate also is converted to acetyl-CoA for fatty acid synthesis and oxaloacetate for the synthesis of nonessential amino acids (Fig. 1). The pyruvate generated in these reactions can be used to produce glucose (reverse glycolysis), which enters the PPP, maximizing the production of NADPH. The glutamate can be converted to aspartate, which contributes to nucleoside synthesis. The excessive quantities of glutamine taken into and used by the cell results in the secretion of alanine and ammonium, which together with lactate bathe the extracellular matrix.

Glutamine is also a major cancer cell energy and anabolic substrate that requires functional mitochondria. However, Warburg's hyphothesis had its basis in the theory that glycolysis is predominately used in cancer cells because of a dysregulation of mitochondrial oxidative phosphorylation. Research has shown that most cancer cells do not have defects in mitochondrial metabolism except for rare mutations in succinate dehydrogenase (SDH) or fumarate hydratase (FH), both enzymes of the TCA cycle and both initiating events of familial paraganalioma or leiomyoma and of papillary renal cell cancer.

Thus, cancer cells maximize their ability to synthesize substrates for membranes, nucleic acids, and proteins. This results in increased cell mass and allows cell division when needed. This cannot be accomplished without large amounts of energy (ATP), which are obtained by increasing the use of glucose and glutamine many fold. Penalties for this increased flux are an increase in oxidative intermediates, an altered redox potential, and excessive ROS. The metabolic response is to focus reactions on producing NADPH, a coolant that feeds into many chemical systems that reduce the ROS activity. This high rate of glucose and glutamine flux must be handled by increased metabolic enzyme levels or increased enzyme activities. Remarkably, this is accomplished by oncogenes and tumor suppressor genes as well as regulators of the response to hypoxia. All of these metabolic pathways (TCA, PPP, and glycolysis) contain complex regulatory circuits at the levels of transcription, mRNA splicing, translation, and small molecule feedforward and feedback loops. A deeper understanding of these regulatory pathways that connect the genetics of cancer to the biochemical metabolic pathways may reveal selected metabolic processes that might be good drug targets for slowing or reversing cancers.

Over the past 10 years, evidence has accumulated that the oncogenes myc, nuclear factor κB (NF-κB), AKT, and the tyrosine kinase receptors (epidermal growth factor, EGF; insulin-like growth factor 1, IGF-1; Her-2; etc.), which turn on Ras, RAF–mitogen-activated protein kinase (MAP kinase), and the phosphatidylinositol 3-kinases (PI3Ks) and mammalian target of rapamycin (mTOR) pathways (Fig. 2) along with hypoxia-induced factor (HIF), can stimulate the transcription of a number of genes that encode the proteins that mediate the glycolysis and glutaminolysis pathways (Fig. 1). The rate of glycolysis can vary over 100-fold. High AKT and mTOR activities result in high HIF activity. Both the myc and HIF-1 transcription factors increase the rate of transcription of some of the GLUT transporters and hexokinase 2, enhancing both glucose uptake and its retention in the cell (*11*). HIF increases the rate of transcription of over 100 genes, resulting in angiogenesis, cell migration, cell survival, and energy metabolism.

Among the HIF-regulated genes are 9 of the 10 enzymes that function in glycolysis (*12*, *13*). HIF is regulated by the cellular hypoxia response. Acute hypoxia stabilizes the HIF-1α and HIF-2α proteins, which form dimers with HIF-1β and HIF-2β. The stability of HIFα subunits is controlled by HIF prolyl-hydroxylases (PHDs), which use O_2 and α-ketoglutarate to convert a prolyl residue to hydroxproline plus succinate and CO_2 (*14*, *15*). The hydroxyl-proline residues are bound by the von Hippel–Lindau protein complex (VHL tumor suppressor lost in some types of cancers) containing an E3 ubiquitin ligase, which results in the HIFα subunit being polyubiquitinated and degraded (*16*). The absence of oxygen stabilizes the HIF transcription factor. In addition, lactate dehydrogenase A and pyruvate dehydrogenase kinase are transcriptionally regulated by HIF, both of which keep pyruvate away from the mitochondria. The loss of PTEN (phosphatase and tensin homolog, a tumor suppressor gene) and concurrent increase of AKT-1 and mTOR lead to HIF activation and the Warburg effect (Fig. 2). The myc transcription factor activates the transcription of more than 1000 genes involved in all phases of cell growth and metabolism. Myc enhances the transcription of glutaminase-1, the first enzyme in glutaminolysis producing glutamate (*17*), and it transcribes the ribosomal RNA genes and the ribosomal protein genes, increasing the rate of protein synthesis and mass of a cell (*18*). Myc also regulates glutaminolysis at the microRNA (miRNA) level by transcriptionally repressing miR-23a and miR-23b, which results in greater expression of their target protein, mitochondrial glutaminase-1, and thus up-regulation of glutamine catabolism.

Similarly, the loss of p53 functions lead to the Warburg effect (Fig. 2). The p53 protein represses the transcription of the GLUT 1 and 4 transporters (*18*). The p53 protein induces the transcription of the TIGAR gene, which lowers the intracellular concentrations of fructose 2,6 bisphosphatase (FBPase) and thus decreases glycolysis by diverting glucose through the PPP (*19*) (Fig. 1). TIGAR also has functional similarities to the bisphosphate domain of PFK-2/FBPase-2 in regulating glycolysis, ROS levels, and apoptosis and is structurally similar to FBPase2. The activation of p53 also increases the ubiqutination of phosphoglycerol mutatase, which decreases the activity of this glycolytic enzyme. p53 increases the use of the TCA cycle and oxidative phosphorylation. The p53 protein enhances the transcription of the gene for synthesis of cytochrome c oxidase 2 (SCO2), which, along with synthesis of cytochrome c oxidase 1 (SCO1), assembles into oxidative phosphorylation complexes (*20*). Cells with mutant p53 have compromised oxidative phosphorylation chains. p53 also promotes synthesis of a number of proteins that reduce the high ROS load in cells. Sestrins 1 to 4 are p53-regulated genes and produce proteins that react with and neutralize ROS (*21*). p53 also regulates the p21 gene, and the p21 protein binds to and stabilizes the Nrf2 transcription factor, which regulates a set of complex responses to altered redox potentials and high ROS. P53 transcribes the glutaminase 2 gene, a nuclear gene that produces a glutaminase localized in the internal compartment of mitochondria (*22*, *23*). Unlike glutaminase 1, glutaminase 2 converts glutamine to glutamate, which can be used to enhance the rate of the TCA cycle and oxidative phosphorylation (*22*, *23*). Thus, these two glutaminases, regulated by myc (glutaminase 1) and p53 (glutaminase 2), have opposite effects on the cell. Just why this is the case remains to be elucidated. An activated p53 protein also inhibits the activities of the phosphatidylinositol-3 kinase (PI3K)–AKT and mTOR pathways (Fig. 2). P53 regulates the transcription of four genes, PTEN, IGF-binding protein-3 (IGF-1BP-3), tuberous sclerosis protein 2 (TSC-2), and the beta subunit of AMP-activated protein kinase (AMPK), which all negatively regulate AKT kinase and mTOR (*24*, *25*). In addition, sestrins 1 and 2, which are p53-regulated genes, stimulate AMPK activity (*26*) All of these activities shut down cell growth, decrease the Warburg effect, lower HIF levels, and thus reverse the cancer phenotype. In some cases, this results in a p53-directed apoptosis and the activation of autophagy (*27*).

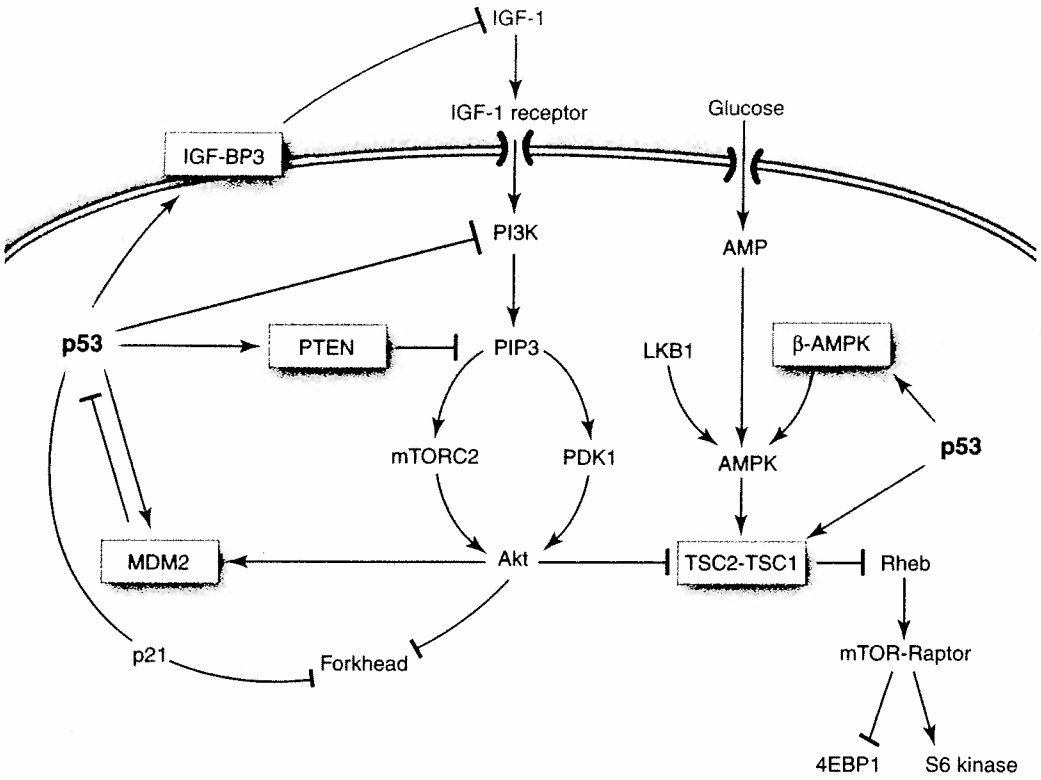

Fig. 2. p53 regulation of PI3K, Akt, and mTOR pathways. p53 functions in a complex network to mediate a cell's adaptation to stress. To do this, p53 regulates the transcription of four genes, PTEN, IGF-BP3, TSC2, and AMPKβ, which then all negatively regulate Akt kinase and mTOR, leading to a decrease in cell growth and a reversal of the cancer phenotype. Coordinately, there is an inhibition of proliferation that is promoted through an activation of p53 and LKB1. In addition to slowing cell growth, p53-dependent inhibition of the mTOR pathway promotes autophagy, a way of helping cells survive. 4EBP1, 4E-binding protein 1; IGF-1 receptor, insulin-like growth factor–1 receptor; LKB1, liver kinase B1; MDM2, murine double minute 2; PDK1, phosphoinositide-dependent kinase-1; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Raptor, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; S6 kinase, ribosomal protein S6 kinase; TSC, tuberosclerosis complex.

A number of mutations in genes that encode enzymes in the TCA cycle have been shown to lead to some types of cancers. Mutations in succinate dehydrogenase and fumarate hydratase alter the complex 2 oxidative phosphorylation chain, which generates reduced flavine adenine dinucleotide (FADH2). These mutations force a switch to the Warburg effect and contribute to selected inherited and sporadic cancers (*27*). There is some evidence that these mutations result in the inactivation of the PHDs, leading to increases in HIF-1 and an enhanced glycolytic pathway. In glioblastoma multiforme, up to 12% of these tumors have spontaneous point mutations in the gene for cytosolic isocitrate dehydrogenase 1 (IDH1) (*28*). This enzyme converts isocitrate to α-ketoglutarate, generating NADPH. Likewise, mutations have been observed in IDH2 at residue Arg^{172}, in the active site, in patients of low-grade gliomas (*29*) and acute myeloid leukemia (AML) (*30*), as well as other diseases (*31*). The somatic mutations in IDH1 and IDH2 identified in gliomas and AML result in a new ability of the enzyme to catalyze the NADH-dependent reduction of α-ketoglutarate to 2-hydoxyglutarate, an oncometabolite that can be a correlative marker for mutations occurring in isocitrate dehydrogenase enzymes (*32*). Just why this is so strongly selected for in these tumors may well be more complex than simply generating more NADPH.

The large number of genetic alterations observed in human cancers in the oncogenes and tumor suppressor genes involved in the IGF-1/mTOR pathways (Fig. 2) suggest that drugs may be developed that alter the Warburg effect and some of its consequences. Inhibitors of TOR complex1 (TORC1), which controls protein synthesis and cell cycle progression, are already approved for use in selected cancers. TORC1 is regulated by AMPK, which measures ATP/AMP ratios and nutrient availability. Metformin, which is used as a treatment for type 2 diabetes, stimulates AMPK. Diabetic patients treated with metformin have lower incidences of cancer than diabetics not treated with this drug (*33*, *34*). Indeed, metformin acts as a synthetic lethal drug on cells in culture that contain p53 mutations, demonstrating the close interactions between these two pathways (*35*). The inhibition of lactate dehydrogenase in cancer cells slows their growth, suggesting the importance of making and secreting lactate from cancer cells. Most hepatocellular carcinomas have lost the expression of glutaminase-2 in mitochondria, even though most of these tumors do not contain p53 mutations (p53 regulates this gene). Returning a cDNA for glutaminase-2 to these cells in culture and expressing this protein, which enhances the use of the TCA cycle, inhibits cell division (*22*, *23*). Thus, glutaminase-2 is acting like a tumor suppressor gene in these situations. Indeed, a wild-type p53 gene and protein are required for efficient mitochondrial DNA replication and mitochondrial maintenance in cells (*36*). These types of observations suggest that the extensive alterations of metabolic processes in cancer can contribute to the phenotypes of the tumor cells and as such are themselves causal (necessary but not sufficient) for these cancers.

Conclusions

The observations and ideas reviewed here suggest a unity in the genes and pathways involved in several diseases. The interrelationships of the p53, AKT, and mTOR pathways (Fig. 2) bring together stress responses and diabetes. Indeed, p53 in adipose tissue can regulate insulin resistance (*37*). There is a similar overlap among several genes whose mutations predispose an individual to Parkinson's disease and the functions of those genes in the p53, AKT, and mTOR pathways (*38*). The connections among chronic inflammatory responses of the immune system, with the activation of NF-κB and its associated metabolic changes (Warburg effect) and PET scan–positive cells, and the formation of cancers of those cells are well established (*39*). It should not be surprising to observe such a central role of metabolic processes in many disorders and the integration of metabolic pathways with many diverse signal transduction pathways. Metabolic pathways comprise an evolutionarily conserved underlying feature for most functions of a cell and an organism.

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REVIEW

Autophagy and Metabolism

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Autophagy is a process of self-cannibalization. Cells capture their own cytoplasm and organelles and consume them in lysosomes. The resulting breakdown products are inputs to cellular metabolism, through which they are used to generate energy and to build new proteins and membranes. Autophagy preserves the health of cells and tissues by replacing outdated and damaged cellular components with fresh ones. In starvation, it provides an internal source of nutrients for energy generation and, thus, survival. A powerful promoter of metabolic homeostasis at both the cellular and whole-animal level, autophagy prevents degenerative diseases. It does have a downside, however—cancer cells exploit it to survive in nutrient-poor tumors.

Living organisms from yeast to humans are capable of eating parts of themselves in order to survive. This involves the degradation of cellular components, either because they are deleterious (e.g., damaged organelles and microbial invaders) or because the resulting breakdown products are needed to support metabolism. This process was aptly termed autophagy from the Greek "auto" or oneself and "phagy" or to eat. It has gained attention recently as an essential contributor to human health and disease.

There are several forms of autophagy, each of which involves delivering intracellular cargo to lysosomes for degradation. The predominant form, macroautophagy (autophagy hereafter), produces vesicles called autophagosomes that capture and deliver cytoplasmic material to lysosomes (*1*). The autophagy-related genes (the *atg* genes) are conserved from yeast to mammals and regulate the cannibalism of intracellular cytoplasm, proteins, and organelles.

Autophagy is the only mechanism to degrade large structures such as organelles and protein aggregates. In the absence of stress, basal autophagy serves a housekeeping function. It provides a routine "garbage disposal" service to cells, eliminating damaged components that could otherwise become toxic. Such cellular refreshing is particularly important in quiescent and terminally differentiated cells, where damaged components are not diluted by cell replication. In starvation, autophagy provides a nutrient source, promoting survival. Autophagy is induced by a broad range of other stressors and can degrade protein aggregates, oxidized lipids, damaged organelles, and even intracellular pathogens. Although it is not always possible to resolve the metabolic and garbage disposal roles for autophagy, it is clear that autophagy prevents disease. Defects in autophagy are linked to liver disease, neurodegeneration, Crohn's disease, aging, cancer, and metabolic syndrome.

Process of Autophagy

A series of protein complexes composed of *atg* gene products coordinate the formation of autophagosomes. The Atg1/ULK1 complex (Atg1 in yeast and ULK1 in mammals) is an essential positive regulator of autophagosome formation (*1*). When nutrients are abundant, binding of the ULK1 complex by the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibits autophagy. mTORC1 is an important regulator of cell growth and metabolism. It is composed of five subunits that include Raptor, which binds ULK1, and mTOR, a serine-threonine kinase. By phosphorylating ULK1 and another complex member (the mammalian homolog of yeast Atg13), mTOR inhibits autophagy initiation. In starvation, mTORC1 dissociates from the ULK1 complex, freeing it to trigger autophagosome nucleation and elongation.

Autophagosome nucleation requires a complex containing Atg6 or its mammalian homolog, Beclin 1, that recruits the class III phosphatidylinositol 3-kinase VPS34 to generate phosphatidylinositol 3-phosphate (*2*). Expansion of autophagosome membranes involves two ubiquitin-like molecules, Atg12 and Atg8 (called LC3 in mammals), and two associated conjugation systems. The E1-like Atg7 and E2-like Atg10 covalently link Atg12 with Atg5, which together bind Atg16L1 to form pre-autophagosomal structures. In the second ubiquitin-like reaction, LC3 is cleaved by the protease Atg4. Phosphatidylethanolamine is conjugated to cleaved LC3 by Atg7 and a second E2-like enzyme, Atg3, and this lipidated LC3-II associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until after fusion with lysosomes and is commonly used to monitor autophagy.

The process beginning with the Beclin 1 complex gives rise to nascent autophagosome membranes. These membranes assemble around cargo, encapsulating the cargo in a vesicle that subsequently fuses with a lysosome, generating an autolysosome. The contents are then degraded by proteases, lipases, nucleases, and glycosidases. Lysosomal permeases release the breakdown products—amino acids, lipids, nucleosides, and carbohydrates—into the cytosol, where they are

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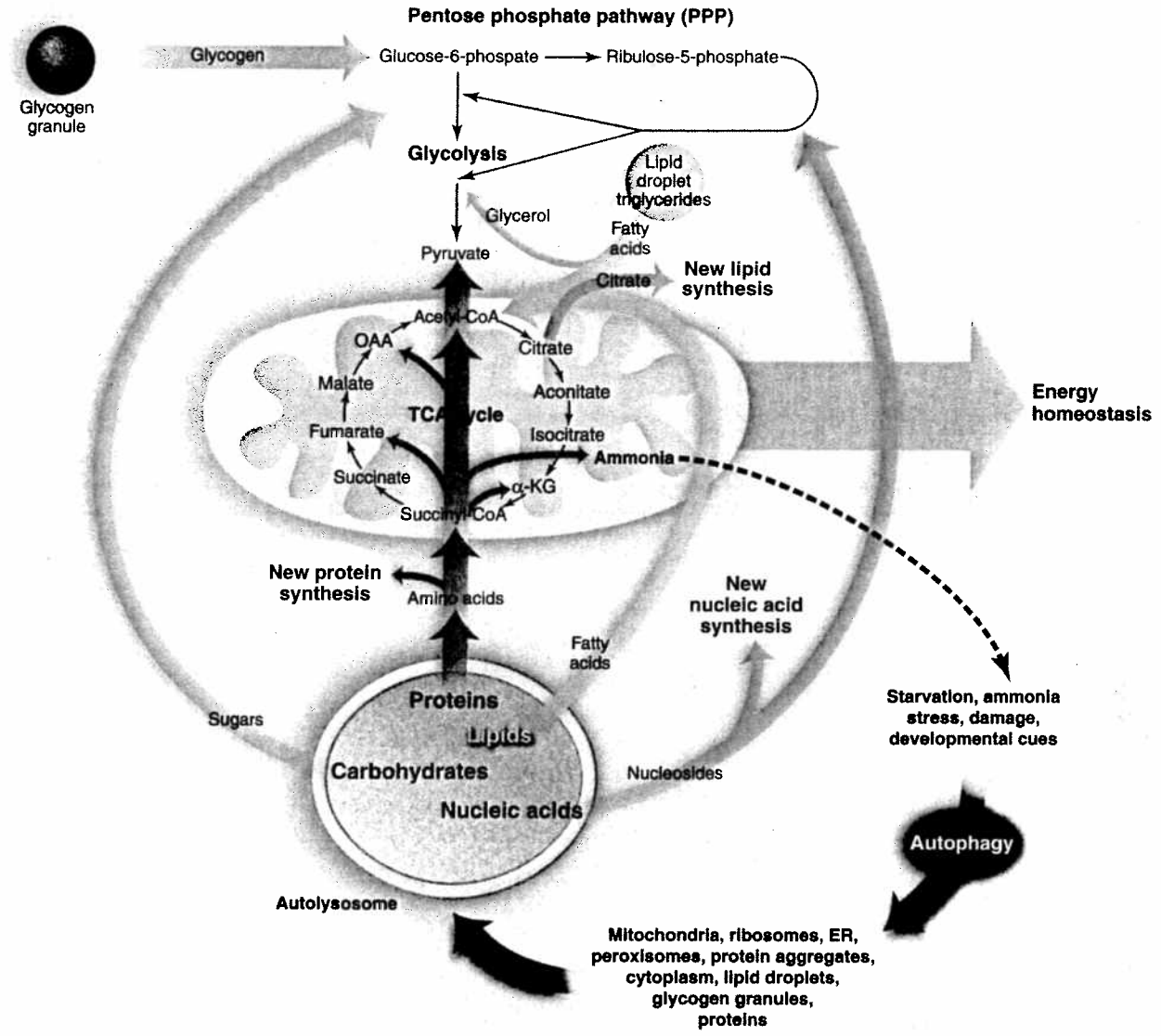

Fig. 1. Use of the products of autophagy. Multiple forms of stress activate autophagy (bottom right). Degradation of proteins, lipids, carbohydrates, and nucleic acids liberates amino acids, fatty acids, sugars, and nucleosides that are released into the cytoplasm for reutilization. Sugars (blue lines), including glucose released from glycogen granules by glycogenolysis or autophagy, are catabolized by glycolysis and the PPP to generate ATP, and pyruvate for subsequent TCA cycle metabolism. Nucleosides (green lines) are used for new nucleic acid synthesis and catabolized by the combined action of the PPP and glycolysis. Amino acids (purple lines) are used as building blocks for new protein synthesis, for ATP production by central carbon metabolism, and (in liver) as substrates for gluconeogenesis (Fig. 3). They also can be combined to yield citrate, which drives lipid synthesis and membrane biogenesis. Catabolism of amino acids yields ammonia, an activator of autophagy (dotted line). Fatty acids (yellow lines) from lipolysis or from autophagy of membranes or lipid droplets yield acetyl-CoA, which feeds the TCA cycle, supporting ATP production and citrate generation. OAA indicates oxaloacetate; α-KG, α-ketoglutarate; and ER, endoplasmic reticulum.

available for synthetic and metabolic pathways (Fig. 1).

Substrates of Autophagy

Autophagy can be nonselective or selective. Nonselective, bulk degradation of cytoplasm and organelles by autophagy provides material to support metabolism during starvation. It also contributes to extensive tissue remodeling, as in *Drosophila* morphogenesis (*3*). Whether mechanisms exist to prevent bulk autophagy from consuming essential components, such as a cell's final mitochondrion, remains unclear, and in some cases such consumption may lead to cell death.

Selective autophagy of proteins and of organelles such as mitochondria (mitophagy), ribosomes (ribophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy), and lipids (lipophagy) occurs in specific situations. In mammals, the signal for targeting proteins for degradation by either the autophagy or proteasome pathways is ubiquitination. Many proteins accumulate in autophagy-defective mammalian cells, indicating that autophagy has a major role in controlling the cellular proteome and that proteasome-mediated degradation cannot compensate for defective autophagy (*4*). To target proteins for autophagic degradation, ubiquitin on modified proteins is recognized and bound by autophagy receptors, such as p62 or Nbr1, which

interact with LC3 to deliver cargo to autophagosomes (*1*).

Mechanisms regulating selective autophagy of organelles are more elaborate. In yeast, Atg32 localized in mitochondria is a receptor that interacts with Atg8 and Atg11 to produce selective mitochondrial autophagy (*5*, *6*). In mammals, autophagy of depolarized mitochondria, which protects cells from toxic reactive oxygen species, is initiated by Pink1-dependent mitochondrial translocation of Parkin. This is followed by ubiquitination of mitochondrial proteins and recruitment of p62 to direct mitochondria to autophagosomes (*7*, *8*). The pruning of damaged mitochondria by autophagy has two homeostatic functions. The first is limiting oxidative damage. The second is in maintaining a functional mitochondrial pool.

Regulation of Autophagy

Cells integrate information regarding nutrient availability, growth factor and hormonal receptor activation, stress, and internal energy through an elaborate array of signaling pathways (Fig. 2). In mammals, insulin—the master hormone of the fed state—blocks autophagy.

A major intracellular hub for integrating autophagy-related signals is mTORC1 (*9*). In the presence of abundant nutrients and growth factors including insulin, mTORC1 promotes cell growth and metabolic activity while suppressing the ULK1 complex and autophagy. In deprivation or stress, numerous signaling pathways inactivate mTORC1 kinase activity. This both suppresses cell growth to reduce energy demand and induces autophagy to enable stress adaptation and survival. A second mTOR complex, mTORC2, positively regulates mTORC1. Upstream of mTORC1 is the cellular energy–sensing pathway controlled by adenosine monophosphate–activated protein kinase (AMPK) (*10*). High concentrations of AMP signal energy depletion, activate AMPK, and inhibit mTORC1, thus promoting autophagy (Fig. 2).

Regulation of autophagy also occurs through the forkhead box or FOXO transcription factors, whose activation leads to transcription of *atg* genes (*11*). Similarly, hypoxia and activation of hypoxia-inducible factors, or HIFs, induces the transcription of mitophagy-specific genes and mitophagy (Fig. 2) (*12*). Less well-characterized mTOR-independent regulators of autophagy also exist. One is ammonia, a by-product of amino acid catabolism, which stimulates autophagy, likely in poorly perfused tissues and tumors (*13*). Glucagon, a predominant hormone of the fasted state, also triggers autophagy in the liver. Adrenergic receptor activation, which like glucagon activates adenylate cyclase and cyclic adenosine monophosphate (cAMP) production, also stimulates liver autophagy.

Autophagy and Starvation

All cells have internal nutrient stores for use during starvation. Glycogen and lipid droplets are overtly designed for this purpose. Their contents are accessed primarily through the actions of dedicated enzymes, such as glycogen phosphorylase and hormone-sensitive lipase. Many other cellular components have a dual function as nutrient stores. For example, ribosomes occupy ~50% of the dry weight of rapidly growing microbes. In addition to enabling rapid protein synthesis when nutrient conditions are favorable, this provides a store of amino acids for proteome remodeling when conditions turn for the worse. Autophagy has a key role in providing access to such undedicated nutrient stores.

Limitation for any of the major elemental nutrients triggers autophagy in yeast, with nitrogen limitation the strongest stimulus (*14*). When nitrogen is removed, yeast defective in autophagy become severely depleted of internal amino acids. This precludes the synthesis of proteins important for surviving nitrogen starvation and accelerates cell death (*15*). Thus, autophagy provides the primary route to nitrogen during starvation.

Unlike microbes, mammalian cells benefit from a relatively constant nutrient environment. Nevertheless, autophagy can support mammalian cells through nutrient deprivation. For example, in lymphocytes, the ability to consume environmental nutrients is growth factor–dependent. In the absence of growth factor stimulation, energy charge is maintained through autophagy, with cells shrinking ~50% in size over 3 months of self-cannibalization (*16*).

At the organismal level, autophagy is required at multiple stages of mammalian development. The first directly follows oocyte fertilization, with autophagy essential to feed the developing embryo before it gains access to the maternal blood supply. Autophagy-defective embryos fail to reach the blastocyst stage (*17*). Maternally supplied autophagy proteins enable autophagy-deficient offspring to complete embryogenesis, revealing a second requirement for autophagy: when access

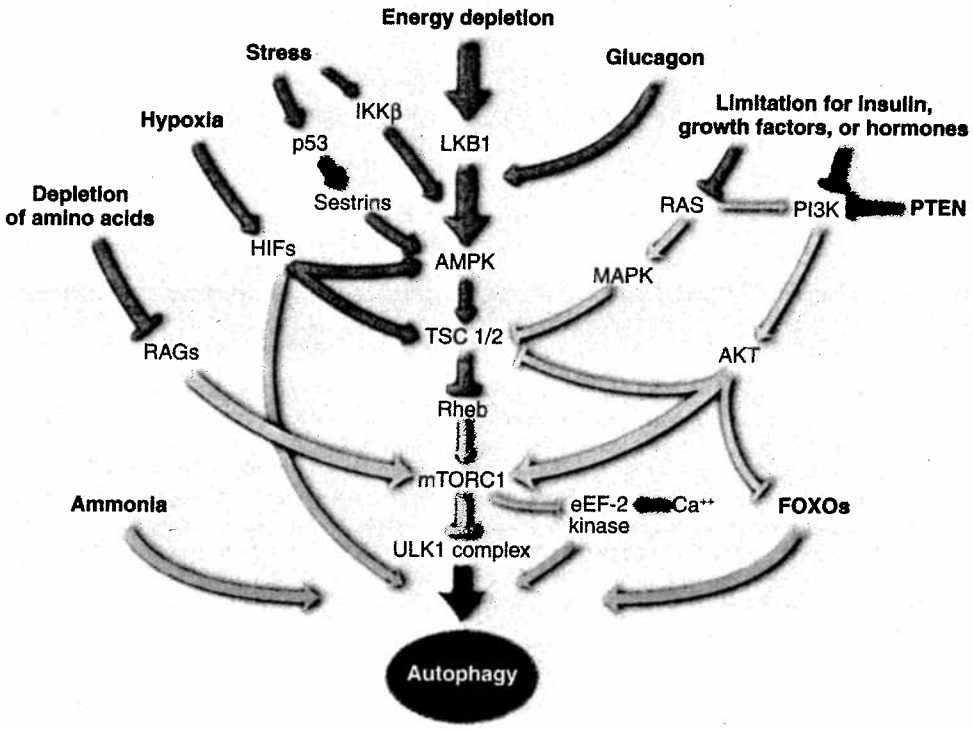

Fig. 2. Signaling pathways that regulate autophagy. Common nutrient, growth factor, hormone, and stress signals that regulate autophagy. Purple lines depict events that positively regulate autophagy. Yellow lines depict those that negatively regulate autophagy. Many pathways converge on the AMPK-mTORC1 axis. Green lines depict pathways that are mTOR-independent. Note that all input signals are framed as autophagy activators; thus, they include limitation for growth factors and nutrients. IKKβ, inhibitor of nuclear factor κB kinase β; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; MAPK, mitogen-activated protein kinase; TSC1/2, tuberosclerosis complexes 1 and 2; and EF, elongation factor.

to the maternal blood supply is suddenly lost due to birth. Autophagy-defective pups die within 24 hours of delivery. Both circulating and tissue amino acid levels are reduced, and AMPK is activated in the heart, which shows electrocardiographic changes analogous to those observed with severe myocardial infarction (*18*).

In adult starvation, autophagy also has a central role, increasing within 24 hours in liver, pancreas, kidney, skeletal muscle, and heart; the brain is spared (*19*). Pharmacological blockade of autophagy results in cardiac dysfunction early in starvation (*20*). Although autophagy levels return to normal in liver 2 days into starvation, it remains increased in both cardiac and skeletal muscle. Liver mass, however, persistently falls faster than muscle or total body mass. This decline is consistent with a failure of biosynthesis to balance basal consumption of liver by autophagy (*21*). As liver mass falls, breakdown of muscle and adipose tissue feeds the liver, which exports glucose and ketone bodies required by the brain (Fig. 3).

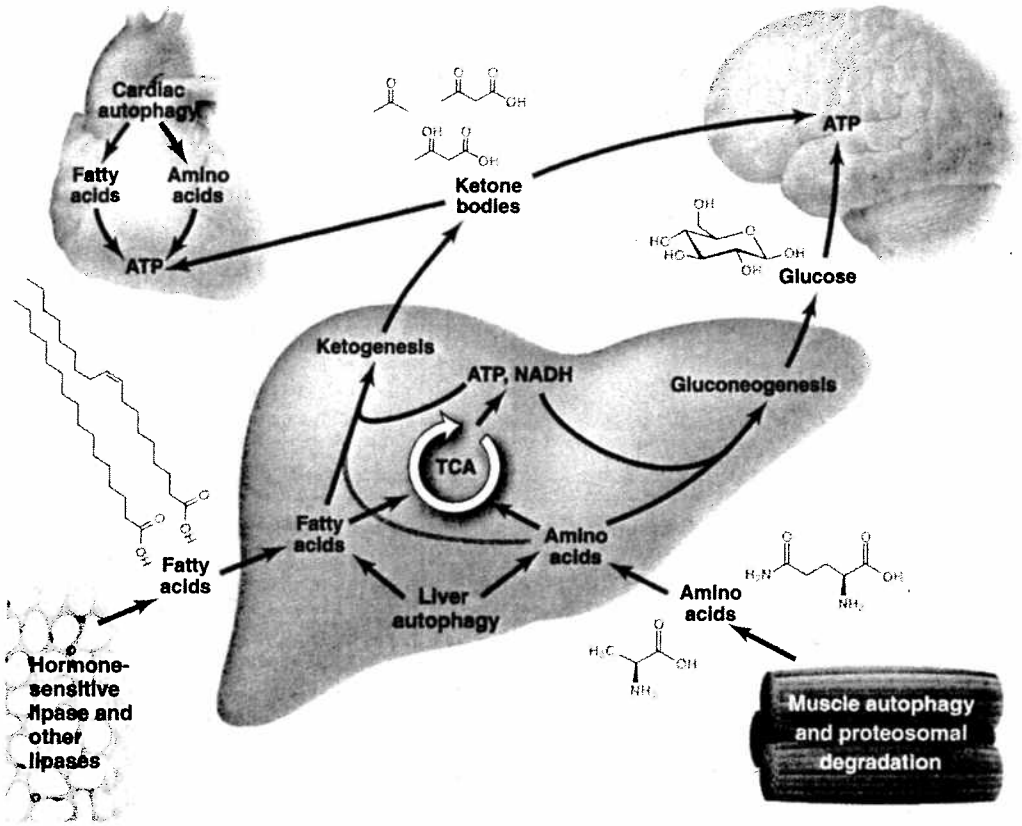

Fig. 3. Role of autophagy in adult mammalian starvation. Depicted pathways predominate after depletion of glycogen stores, typically ~12 hours into starvation. Autophagy in liver and heart (but not brain) generates fatty acids and amino acids, which are catabolized to yield energy. In the liver, this energy drives gluconeogenesis and ketogenesis. Amino acids are substrates for both ketogenesis and gluconeogenesis; acetyl-CoA from fatty acids is only for ketogenesis. As starvation continues, degradation of adipose and muscle play an increasing role in supplying substrates to the liver, which exports glucose and ketone bodies to feed the brain. The relative importance of ketone bodies increases in prolonged starvation. NADH, reduced form of nicotinamide adenine dinucleotide.

Use of Metabolites Released by Autophagy

The breakdown products derived from autophagy have a dual role, providing substrates for both biosynthesis and energy generation (Fig. 1). In terms of biosynthesis, the abundance of ribosomal (relative to messenger) RNA makes transcriptome remodeling straightforward. In contrast, proteome remodeling demands copious amino acids, and a major role of autophagy is to provide them.

In addition to providing anabolic substrates, nucleosides and amino acids can be catabolized for energy generation. RNA breakdown yields nucleosides, which are degraded to ribose-phosphate. Six ribose-phosphate molecules are energetically equivalent to five glucose-phosphates, and, like glucose-phosphate derived by glycogen breakdown, they can yield adenosine triphosphate (ATP) either aerobically or anaerobically. In contrast, amino acids, like lipids, yield ATP only through oxidative phosphorylation (Fig. 1). The catastrophic effects of ischemia are a consequence of the relative paucity of nucleic acids and glycogen combined with the inefficiency of anaerobic glycolysis. Oxygen is the one nutrient that autophagy cannot provide.

In addition to being directly catabolized to yield energy, the liver can convert nucleosides, amino acids, and lipids into glucose and ketone bodies for distribution elsewhere in the body (Fig. 3). Ribose-phosphate from nucleosides can be converted to glucose through the nonoxidative pentose phosphate pathway (PPP). Amino acids feed into central metabolism at multiple points, including pyruvate, tricarboxylic acid cycle (TCA) cycle intermediates, and acetyl–coenzyme A (CoA) (Fig. 1). Pyruvate and TCA cycle intermediates are substrates for gluconeogenesis. In contrast, mammals cannot convert acetyl-CoA into glucose. Because lipid degradation yields mostly acetyl-CoA, ketone bodies are essential for feeding the brain and other vital tissues during prolonged starvation.

Autophagy as a Regulator of Metabolism

Autophagy is important for regulating cellular metabolic capabilities. A striking example comes from yeast capable of living off of methanol or fatty acids, substrates that are burned in peroxisomes. When more appealing forms of carbon become available, the peroxisomes are no longer required and are cleared by autophagy (*22*). The function of autophagy in removing unneeded peroxisomes is conserved in mammals. Peroxisomes are induced in liver by various hydrophobic chemicals, known collectively as peroxisome proliferators. Removal of peroxisome proliferators leads to restoration of normal peroxisome abundance through autophagy (*22*).

Autophagy also regulates the abundance of liver lipid droplets by their constitutive degradation (*23*). Defective autophagy in mice leads to larger and more plentiful lipid droplets, increased concentrations of hepatic triglycerides and cholesterol, and increased gross liver size. Lipophagy is selectively decreased by free fatty acids in vitro and by a high-fat diet (*23*). Thus, in addition to promoting lipid droplet growth, free fatty acids may impair lipid droplet breakdown. Because lipophagy releases free fatty acids, its suppression by them is a case of one of the most prevalent regulatory motifs in metabolism: feedback inhibition. But this

feedback mechanism may backfire in the case of a chronic high-fat diet or obesity.

In contrast to the role of autophagy in clearing lipid droplets from the liver, autophagy is required for the production of the large lipid droplets characteristic of white adipose tissue (*24*, *25*). White adipose refers to the canonical fat storage tissue that expands in obesity; this is in contrast to brown adipose, a mitochondria-rich tissue that catabolizes glucose and lipids to generate heat rather than ATP. Brown adipose tissue contains uncoupling protein 1, which allows protons to leak across the inner mitochondrial membrane, short-circuiting oxidative phosphorylation. Inhibition of autophagy blocks white adipocyte differentiation, and adipose-specific knockout of *atg7* results in mice whose white adipocytes manifest features typical of brown adipose tissue. Consistent with the rapid energy burning of brown adipocytes, these mice are lean; however, they are not healthy—when fed either a regular or high-fat diet, they are at increased risk of early death (*24*).

Autophagy also contributes to both insulin secretion and physiological sensitivity to the hormone. It is essential for the health of pancreatic β cells and for the expansion of β-cell mass that occurs in response to a high-fat diet (*26*, *27*). In liver, defective autophagy leads to insulin resistance (*28*). For reasons that are not yet fully clear, hepatic autophagy is decreased in obese mice, and its restoration through retroviral expression of *atg7* ameliorates their insulin resistance.

Overall Energetic Impact of Autophagy

To maintain homeostasis, tissue degradation by autophagy must be balanced by new macromolecule synthesis, which is energetically expensive. Each peptide bond in protein costs four high-energy phosphate bonds, two for tRNA charging and two for ribosome peptide bond formation. Assuming a free energy of ATP hydrolysis of ~50 kJ/mol under physiological conditions, synthesizing 1 g of protein consumes 1.8 kJ of energy. For a typical human, this means that rebuilding 10% of the body's protein content would consume at least 2000 kJ, or 20% of daily dietary energy intake. In vitro estimates of autophagic rates have generally been at or above the 10% per day level, for example, 1% of protein per hour for cultured hepatocytes (*21*). Accordingly, although in vivo rates of autophagy are presumably lower, rebuilding the structures degraded by autophagy may be a major contributor to mammalian caloric requirements. Consistent with this possibility, knockout of p62, which brings cargo to autophagosomes, results in decreased calorie burning and eventually obesity (*29*). During aging, both autophagy and total caloric expenditures decrease in tandem (*30*). The possibility of a causative link, in which decreased autophagy leads to reduced energy burned for self-regeneration, is intriguing. Decreased autophagy in obesity may also contribute to the difficulty of losing weight (*28*).

Autophagy and Disease

Cellular garbage disposal by autophagy prevents the buildup of damaged proteins and organelles that cause chronic tissue damage and disease. Genetic inactivation of autophagy in mice revealed that the type of disease depends on the tissue type. In the brain, autophagy suppresses the accumulation of ubiquitinated proteins, disposes of aggregation-prone proteins and damaged organelles that cause Huntington's and Parkinson's diseases, and prevents neurodegeneration (*31*, *32*). In the liver, autophagy suppresses protein aggregate and lipid accumulation, oxidative stress, chronic cell death, inflammation, and cancer (*4*, *33*). In intestinal Paneth cells, it preserves cellular function, prevents expression of damage and inflammatory markers, and prevents the development of Crohn's disease (*34*). Although the cell-refreshing role of autophagy functions in preventing the above diseases, autophagy's metabolic role may also contribute by ensuring consistent availability of internal nutrients and enabling cells to survive periods of poor external nutrition in good health. Regardless of the underlying mechanism, autophagy stimulation is under consideration for disease prevention. In support of this concept, autophagy mediates the protective effects of dietary restriction on aging-related diseases in model systems (*35*), and autophagy suppression contributes to the deleterious consequences of obesity (*28*). Induction of autophagy may result in the fasting rituals common in many religions as well modern cleansing rituals, producing health benefits.

In contrast to normal cells in tissues, tumors often reside in an environment deprived of nutrients, growth factors, and oxygen as a result of insufficient or abnormal vascularization. Thus, the effects of autophagy in cancer are paradoxical: Although autophagy can prevent initiation of some cancers, it also may support tumor growth. Autophagy localizes to hypoxic tumor regions most distant from nutrient-supplying blood vessels, where it sustains tumor cell survival (*36*). Whether the primary role of autophagy in tumors is to provide metabolic substrates or prevent buildup of damaged components is not yet known. But either way, inhibition of autophagy may suppress the growth of established tumors (*37*).

Many of the pathways that control autophagy are deregulated in cancer (Fig. 2), and cancer therapeutics targeting these pathways activate autophagy. Some do so directly by inhibiting mTOR, whereas others inhibit upstream nutrient or signaling pathways. Cytotoxic cancer therapies activate autophagy, presumably by inflicting damage. The functional role of autophagy in these settings needs to be established. A particularly interesting possibility is that autophagy favors tumor cell survival. If this proves correct, then inhibition of autophagy might synergize with existing cancer treatments (*37*).

Conclusions

Autophagy is a major contributor to cellular metabolism. It provides internal nutrients when external ones are unavailable. It also provides an essential means of refreshing and remodeling cells. As such, it is required for normal development, including that of metabolic tissues such as adipose tissue and pancreatic β cells. In adults, autophagy promotes metabolic homeostasis and prevents degenerative disease and cancer. Once cancer occurs, however, autophagy may contribute to tumor resiliency. Thus, both activation and inhibition of autophagy hold promise for improved treatment of common, devastating diseases.

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REVIEW

Circadian Integration of Metabolism and Energetics

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Circadian clocks align behavioral and biochemical processes with the day/night cycle. Nearly all vertebrate cells possess self-sustained clocks that couple endogenous rhythms with changes in cellular environment. Genetic disruption of clock genes in mice perturbs metabolic functions of specific tissues at distinct phases of the sleep/wake cycle. Circadian desynchrony, a characteristic of shift work and sleep disruption in humans, also leads to metabolic pathologies. Here, we review advances in understanding the interrelationship among circadian disruption, sleep deprivation, obesity, and diabetes and implications for rational therapeutics for these conditions.

The rising and setting of the sun has captivated naturalists interested in the cause of daily rhythmic phenomenon, prompting de Marian to demonstrate the existence of an internal clock in the 18th century by placing the Mimosa plant in a dark box and showing that its leaves continued to open and close every 24 hours. More than two centuries later, genetic studies in fruit flies paved the way for discovery that the circadian clock is encoded by a set of transcriptional activators and repressors that comprise an autoregulatory transcription-translation feedback loop. The circadian clock in mammals is expressed within pacemaker neurons of the suprachiasmatic nucleus (SCN) that in turn maintain proper phase alignment of peripheral tissue clocks present in nearly all cells. Thus, the brain SCN clock provides "standard time" for all peripheral tissue clocks. In experimental models, clock disruption leads to disorders in glucose metabolism, confirming a role for these genes as key regulators of metabolism and supporting the hypothesis proposed by McKnight and colleagues that circadian cycles are intimately interconnected with metabolic cycles (*1*). Accumulating evidence has revealed that multiple clock genes participate in metabolic homeostasis, suggesting that these proteins have evolved overlapping (or convergent) functions both as intrinsic "hands" of the clock and as regulators of metabolism. Although still at an early stage, emerging studies in humans suggest parallels in the role of circadian genes and metabolic homeostasis. At the epidemiological level, it has been suggested that increased activity during what was "rest" time in the premodern world, together with sleep disruption, have been associated with an increased prevalence of obesity, diabetes, and cardiovascular disease, in addition to certain cancers and inflammatory disorders. This review highlights advances in understanding the molecular coupling between metabolic and clock networks and its relevance to gene-environment and brain-behavioral systems important in energy balance and metabolic disease.

Core Transcriptional Components of the Clock and Posttranslational Regulation

Features of the circadian clock in all organisms include its persistence under constant conditions, a periodicity that is temperature compensated, and its entrainment to light from the sun (Fig. 1). In mammals, cell-autonomous circadian clocks are generated by a transcriptional autoregulatory feedback loop composed of the transcriptional activators CLOCK and BMAL1 and their target genes *Period* (*Per*) and *Cryptochrome* (*Cry*), which rhythmically accumulate and form a repressor complex that interacts with CLOCK-BMAL1 to inhibit their own transcription (*2*). This autoregulatory loop is posttranscriptionally regulated by casein kinases (CK1ε and CK1δ), which target the PER proteins for degradation via the Skp1, Cullin1, F-box protein (SCF)/β-TrCP ubiquitin ligase complex, and by adenosine monophosphate (AMP) kinase, which targets the CRY proteins for degradation via the SCF/FBXL3 ubiquitin ligase complex by the 26S proteosome [reviewed in (*2*)] (Fig. 2).

The prevailing model of the circadian clock involves the transcription-translation feedback loop, but less is known about nontranscriptional mechanisms that may generate circadian oscillations. In cyanobacteria, cycles of protein phosphorylation are sufficient to generate biological rhythms in the absence of transcription (*3*). In the mammalian SCN, changes in cyclic AMP levels alter period length, an additional example of posttranslational signaling as a mechanism controlling of circadian cycles (*4*), and recent work has shown that the SCN neuronal coupling network itself has intrinsic oscillatory function that can emerge in the absence of cell-autonomous oscillators (*5*). RNA interference screening of mammalian cells also indicates coupling of the peripheral clock to phosphatidylinositol 3-kinase signaling (*6*). Further research is warranted to elucidate the impact of posttranslational signaling pathways on the core clock and its physiological outputs.

Fibroblast cell lines display ~24 hours oscillation of core clock genes, demonstrating that the clock is expressed not only in neurons but also in peripheral tissues (*7*). Intrinsic oscillation of clocks in liver cells can be entrained by food, whereas oscillation of the brain clock is resilient and entrained primarily by light (*8*). A recurring theme in understanding the coupling between circadian and metabolic systems is the recognition that the two systems are reciprocally regulated; food entrains the liver clock, whereas light acts through the brain clock to control feeding time.

Crosstalk Between the Clock and Metabolic Transcription Networks

Nuclear hormone receptors and the phase alignment of metabolic gene expression cycles. Direct evidence for metabolic input into the core clock includes the finding that the orphan nuclear hormone receptor (NHR) reverse-erb alpha (REV-ERBα) (a repressor) and the opposing retinoic acid orphan receptors (RORα and β) (activators) constitute a short feedback loop controlling *Bmal1* transcription (*9*, *10*) (Fig. 2). Peroxisome proliferator–activated receptor α (PPARα) and the coactivator peroxisome proliferators–activated receptor gamma coactivator 1-α (PGC1α) also modulate *Bmal1* transcription through this feedback loop (*11*), indicating that REV-ERBα is a nodal point for metabolic input into the clock. NHR profiling has revealed rhythmic clustering of these factors in metabolic tissues across the day/night cycle, suggesting extensive coupling between circadian and nuclear receptor signaling networks (*12*). These findings raise the possibility that disruption of NHR cycles may perturb the clock and, conversely, that delay, advance, or reduced amplitude of circadian oscillations may impair NHR function. Knock-in mice of the NHR co-repressor NCor display increased energy expenditure and a shift in the oscillation in the abundance of RNAs encoding oxidative, glycolytic, and respiratory genes, indicating that disruption of the phase of expression of NHRs contributes to metabolic dysregulation (*13*). Mistiming of gene expression rhythms as a cause of metabolic dysregulation has also been suggested by studies in *Rev-erbα* mutant animals, in which a phase shift in oscillating rhythms of metabolic gene transcription, rather than changes in total abundance of RNA, correspond with altered

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energy balance (*14*). Misalignment between gene transcription cycles within metabolic tissues and the behavioral cycle (of fasting and feeding) may be sufficient to alter energy homeostasis. For instance, high-fat feeding provided at the incorrect circadian time leads to greater weight gain in mice than isocaloric feeding at the normal circadian time (*15*).

Direct versus indirect role of clock transcription factors in metabolic gene regulation. It is likely that disruption within the core clock may be transmitted to metabolic outputs through alterations in NHRs or directly by actions of clock activators or repressors. For example, PER2 directly occupies promoters of certain metabolic genes (*16*). Alternatively, the clock activator loop drives D-element–binding protein expression, providing indirect regulation of gluconeogenic genes (*17*). This raises the question as to whether the effects of clock-gene disruption relate to direct alterations in "timing" per se or to indirect effects arising from independent activity of the clock factors on metabolic networks. The dichotomy between circadian versus noncircadian actions of clock proteins may not be fully valid, because the rhythmic abundance in the expression level of these proteins in turn may produce rhythmic changes in metabolism. For example, CRY, a rhythmically expressed clock repressor, modulates gluconeogenesis through interference with glucagon and inhibition of cyclic AMP signaling (*18*). Conceptually, the question of timing versus expression as a cause of metabolic disorders after disruption of clock genes is akin to the difference between a musical performer playing the wrong notes or playing the right notes at the wrong time. One experimental approach to tease apart the role of circadian timing per se in physiology would be to investigate whether physiological defects could be corrected by alignment of the internal period with the external light cycle (i.e., a test of "resonance"). In plants, various period-length mutants have improved photosynthesis and growth when exposed to external light cycles that matched the endogenous circadian period (*19*).

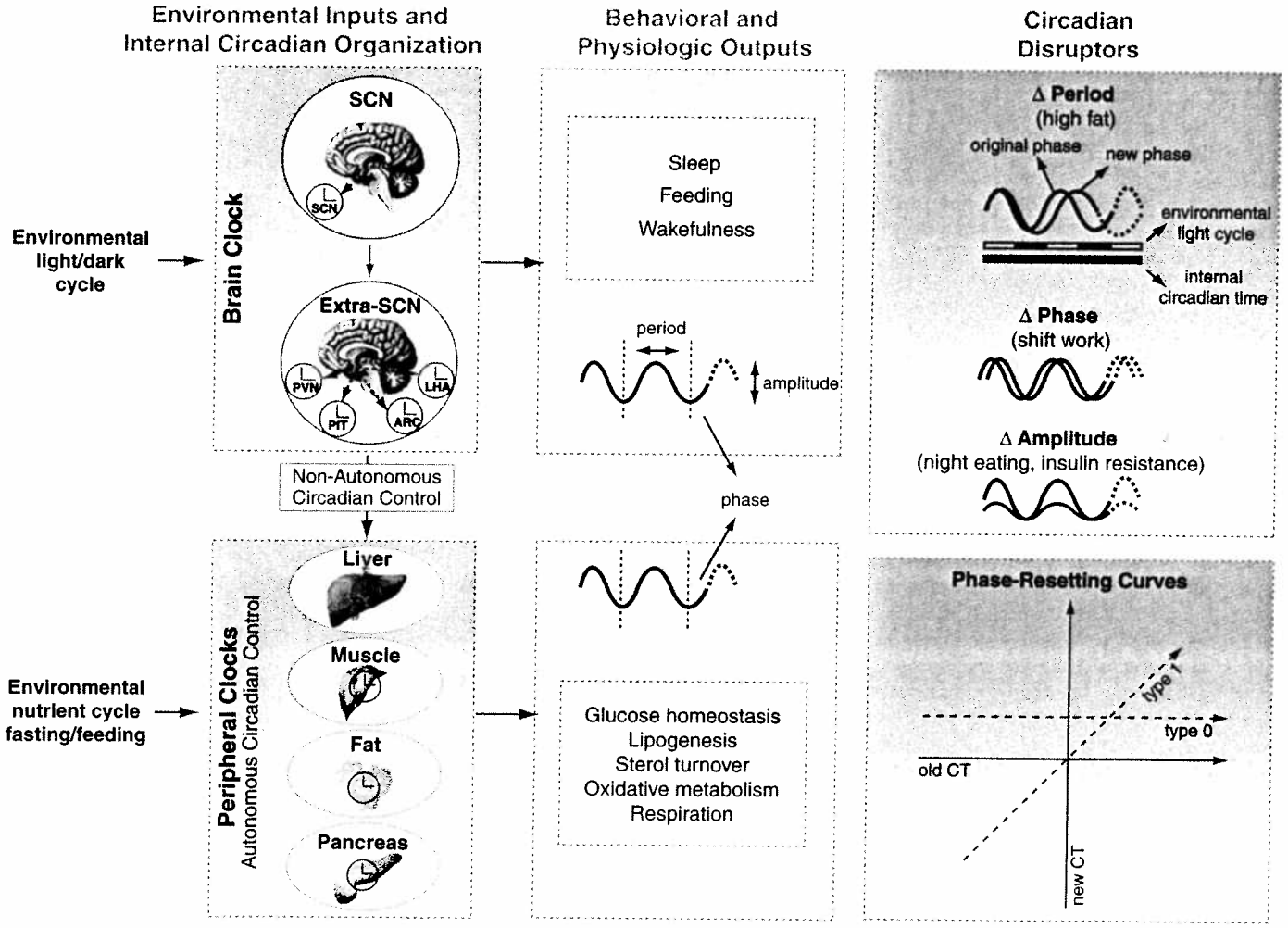

Fig. 1. Central and peripheral clocks coordinate external cues with behavior and metabolic outputs. Light entrains the master pacemaker in the SCN, which in turn synchronizes extra-SCN and peripheral clocks. Brain clock outputs include behavioral rhythms (i.e., sleep and feeding), whereas peripheral clock outputs include metabolic rhythms (e.g., glucose and lipid homeostasis). The hierarchical organization of the mammalian clock is highlighted, with "nonautonomous" regulation of peripheral tissue clocks denoting the regulation of peripheral tissue oscillators through direct neural and humoral signals, and "autonomous" regulation indicating the intrinsic regulation of local cellular oscillators independently of the brain clock. Highlighted to the right are the three possible ways to disrupt the clock by changing period, phase, or amplitude, each of which can trigger disorders of metabolism. Phase resetting can be broadly classified into two groups based on phase response after delivery of the agent at sequential time points across the 24-hour cycle. Type 1 or weak resetting indicates that the slope of the plot relating the new to the old circadian phase is 1 (interventions that cause different phase shifts at different circadian times). Type 0 or strong resetting indicates that the slope of the new to the old circadian phase is 0 (i.e., interventions that cause the same phase at all circadian times). PVN, paraventricular nucleus; PIT, pituitary; ARC, arcuate nucleus; LHA, lateral hypothalamic area.

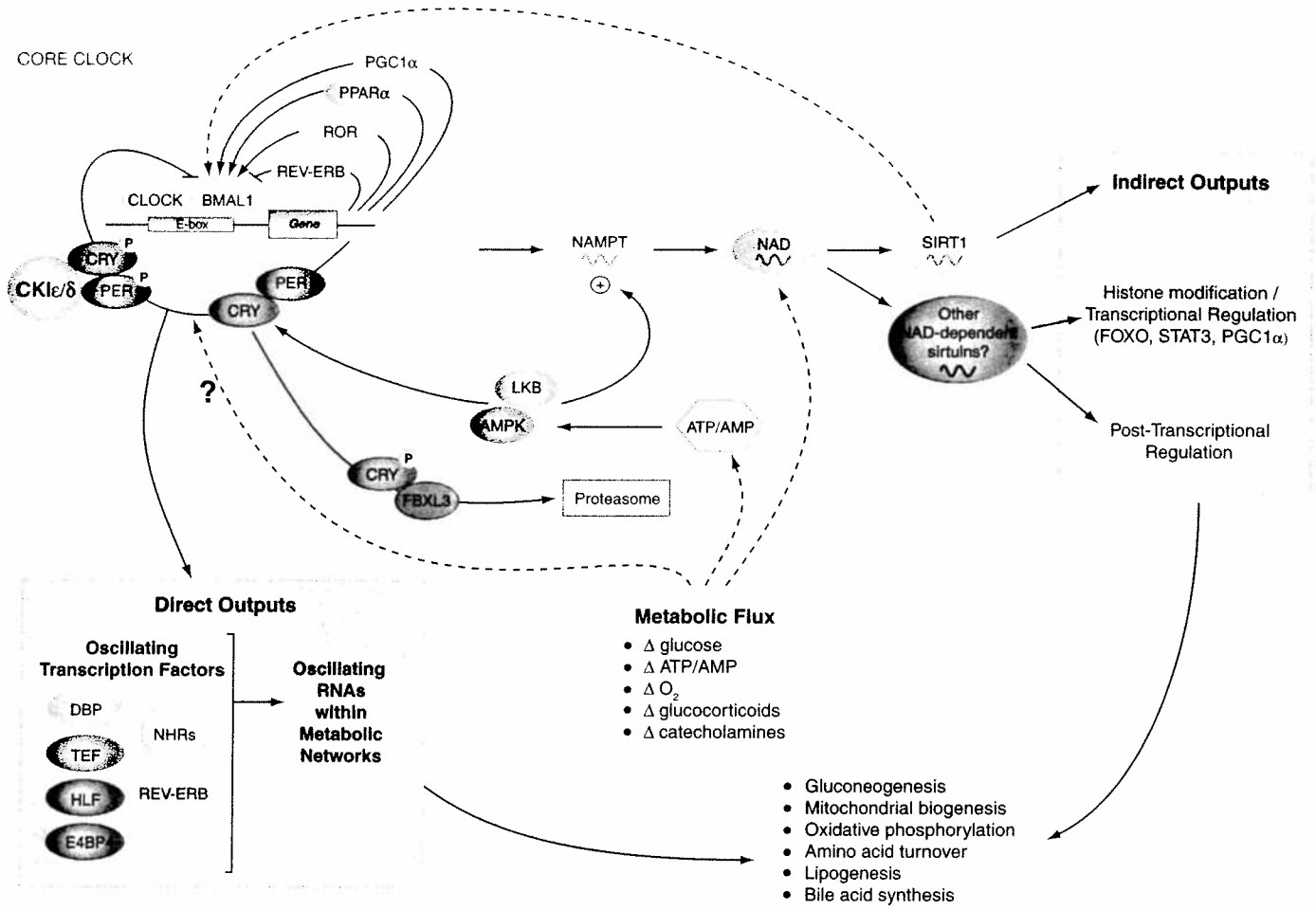

Fig. 2. Direct and indirect outputs of the core clock mechanism. The core clock consists of a series of transcription/translation feedback loops that synchronize diverse metabolic processes through both direct and indirect outputs, including gluconeogenesis and oxidative metabolism. The clock also receives reciprocal input from nutrient signaling pathways (including SIRT1 and AMPK), which function as rheostats to couple circadian cycles to metabolic flux, especially in peripheral tissues.

Reciprocal control of clock by metabolic signaling. An intriguing question remains the extent to which NHRs modulate circadian systems according to changing environmental conditions, such as humoral or nutritional factors. For example, variation in the concentration of glucocorticoid hormone, retinoic acid, heme, and fatty acids affect glucocorticoid receptors (GRs), retinoic acid receptor (RAR), REV-ERBα, neuronal Per-Arnt-Sim (PAS) domain protein 2 (NPAS2), and PPARs. Therefore, variation in cellular concentrations of any one of these ligands might influence *Bmal1* transcription and thereby modulate local cellular circadian rhythms. Within brain, heme and carbon monoxide may modulate NPAS2 activity (*20*), whereas within vascular cells, retinoic acid influences circadian oscillations through activation of RARα and RXRα (*21*). Likewise, rhythmic variation in NHR ligands may exert distinct effects on local tissue clock function at different times in the day/night cycle.

Local differences in NHR expression may give rise to tissue-specific differences in coupling of circadian and metabolic cycles, although this remains to be tested. For example, PER2 forms physical interactions with PPARα and REV-ERBα (*16*), in turn modulating transcription of the gluconeogenic factor *G6pase*. Conversely, oscillation in NHR ligands may affect not only the phase and amplitude of circadian rhythms but also physiological outputs of the circadian system. For example, glucocorticoid receptor binding to the promoter of PER2 modulates leptin production and glucose tolerance (*22*). Nonautonomous signals, such as glucocorticoids or other systemic cues, may have an especially important role in sustaining oscillations of PER2 even in the absence of rhythmic oscillation of CLOCK-BMAL1 (*23*). It may be possible to exploit tissue-specific differences in NHR-clock interactions for therapeutic purposes.

NHRs may also participate in entrainment of central and peripheral clocks. In vertebrates, a hierarchy of signals within SCN pacemaker neurons in the brain and downstream extra-SCN neurons generates entraining cues to maintain phase alignment between oscillators in multiple peripheral tissues. An unresolved question is whether peripheral organ clocks are principally entrained through direct neural wiring or through circulating hormones, such as glucocorticoids (*24*). The impact of glucocorticoids on hepatic entrainment has important implications for health under conditions of circadian misalignment, such as phase resetting during jet lag or shift work (*25*). The finding that liver and brain respond to different entraining signals points toward a possible weak point in the system; conditions such as insulin resistance, where signaling through glucocorticoid, catecholiminergic, or peptideric hormones may be attenuated, may cause misalignment between the phase of central and peripheral oscillators. A related phenomenon is food anticipatory activity (FAA) caused by food presentation at the incorrect circadian time. Although clock-gene function in FAA behavior has been debated, abrogation of melanocortinergic signaling influences this behavior, consistent with a noncircadian timing mechanism (*26*). Finally, body temperature has been shown to be a powerful entraining agent for peripheral oscillators. Indeed, most signals that

synchronize peripheral oscillators affect either body temperature or the heat shock pathway, so this may be a final common pathway for resetting clocks in mammals (*27*).

How Circadian Disruption Causes Metabolic Pathologies: Experimental Genetic Models and Human Clinical Studies

REV-ERB and bile acid synthesis. Further studies on the repressor REV-ERBα have uncovered a connection between the clock and the master pathway of hepatic lipid metabolism involving the sterol regulatory element–binding protein (SREBP). SREBPs control both fatty acid and sterol biosynthesis through modulation of rate-limiting enzymes in these pathways. Using a combination of genetic loss and gain of function approaches, Le Martelot *et al.* observed that the nuclear receptor REV-ERBα controls oscillation in the abundance and activation of the SREBPs through modulation of the enzyme INSIG2 (insulin-induced gene 2), an insulin-responsive factor that regulates SREBP release from the endoplasmic reticulum. REV-ERBα knockout mice develop increased lipogenesis through up-regulation of SREBP1c and SREBP2 target genes independently of nutrient state. In contrast, REV-ERBα–overexpressing mice have decreased SREBP target gene transcription and correspondingly reduced circulating lipid concentrations. The effects of REV-ERBα on bile acid metabolism are mediated through alteration of oxysterol synthesis and liver X receptor (LXR) activity (*14*), although effects of the transcription factor, the small heterodimeric protein (SHP), and E4BP4 (adenoviral E4 protein–binding protein) have also been implicated in this process (*28*). Because REV-ERBα expression is controlled by CLOCK-BMAL1, the rhythmic regulation of bile acid production may represent one of the first direct molecular outputs of circadian clock on metabolic physiology.

Clock network and lipogenesis. In addition to clock control of bile acid synthesis, mounting evidence has implicated both a direct and indirect effect of clock transcription factors on other aspects of lipogenesis. These observations stem from the finding that $Clock^{\Delta 19}$ mutant mice develop hypertriglyceridemia (*29*) due to effects within both enterocytes and liver (*30*). At the level of the intestine, the clock regulates triglyceride packaging into chylomicrons (globules that transport dietary lipids), whereas in the liver, clock disruption triggers lipid accumulation (*30*). The clock-controlled gene *Nocturnin* also affects the interrelated processes of lipogenesis, osteogenesis (bone formation), and energy homeostasis (*31*–*33*). Effects of circadian gene mutations on lipid absorption are strain-dependent in mice, with severe steatorrhea (excess fecal fat) and malabsorption occurring in the ICR (Institute for Cancer Research) strain, thereby masking the effects of the $Clock^{\Delta 19}$ mutation on hepatic triglyceride production, and diet-induced obesity (*34*).

The clock also functions in ultradian variation (cycles occurring multiple times in 24 hours) of endoplasmic reticulum (ER) stress signaling, which in turn modulates SREBP activation through a posttranscriptional pathway (*35*). Rhythmic oscillation of phosphorylation of inositol-requiring enzyme 1α (IRE1α), a transducer of the ER stress response, triggers rhythmic cleavage and translocation of SREBP into the nucleus. The ER stress response detects unfolded or improperly folded proteins; thus, rhythmic activation of IRE1α integrates circadian, stress-signaling, and lipogenic pathways. Indeed, ultradian rhythms within liver appear with a 12-hour periodicity in the expression of many clock-controlled RNAs (*36*) and even in rhythmic oscillation of the metabolite nicotinamide adenine dinucleotide (NAD^+). These shorter cycles are harmonics of the 24-hour cycle and may in turn produce rhythmic patterns in physiologic pathways such as lipogenesis. In *Cry1/Cry2* double-knockout mice, which harbor disruption within the repressor limb of the core clock, loss of circadian oscillation corresponded with constitutive IRE1α activation and accumulation of hepatic lipids. In contrast, in $Clock^{\Delta 19}$ mutant mice, which are deficient in the clock activation limb, there were opposite effects on SREBP activation. The finding that ablation of activators and repressors each produce physiologic effects builds evidence that lipogenesis is driven by the circadian clock rather than an epiphenomenon of clock-gene disruption.

The aforementioned studies in mice also have implications for metabolic functions of the clock in humans, because clock genes oscillate within human adipocytes (*37*) and alterations in clock-gene expression are correlated with obesity (*38*). These findings increase the need to delineate the relationship between chronotype (e.g., whether one is a "lark" or a "night owl"), clock genotype, and metabolic physiology in humans.

Circadian regulation of cardiovascular function, inflammation, and thrombosis. It is axiomatic in clinical medicine that certain cardiovascular catastrophes, including myocardial infarction and thrombosis, cluster early in the morning. Yet, the mechanistic underpinnings of timing in cardiovascular disease are not understood. Many aspects of fatty acid metabolism, a key fuel for cardiac muscle, exhibit strong circadian variation, and clock disruption affects chronotropic function (*39*). Ablation of *Bmal1* also increases the extent of arterial wall lesions after endothelial injury (*40*), suggesting multiple ways through which clock genes may influence susceptibility to myocardial damage. Similarly, autonomic and mineralocorticoid control of vascular tone, factors in cardiovascular disease risk, have been tied to the clock (*41*, *42*). In individuals with metabolic syndrome, one predictor of cardiovascular risk is the absence of normal nocturnal variation in blood pressure, so-called "nondippers" (*43*). Production of the prothrombotic molecule plasminogen activation inhibitor-1 (PAI-1) has been shown to exhibit circadian regulation (*44*, *45*). Thus, inflammation, thrombosis, cardiomyocyte metabolism, vascular tone, and response to vascular injury each represent phenotypes affected by circadian clock function.

Circadian systems in glucose homeostasis and diabetes. Glucose concentrations in the blood are highly rhythmic because of changes in insulin sensitivity and insulin secretory capacity of endocrine pancreas (*46*). Individuals with type 2 diabetes, and even their first-degree relatives not yet affected with the disease, display altered rhythmicity in glucose tolerance (*47*). Although early morning insulin resistance has been ascribed to the surge in growth hormone during slow-wave sleep, rhythmic variation in insulin sensitivity is in part due to autonomic rhythms generated by afferent input from hypothalamus to liver, downstream of the circadian clock (*48*). Ever since the inception of insulin use in clinical practice, recapitulating the endogenous rhythm of insulin production, and achieving a proper match in the variation in insulin requirement throughout the day/night cycle, has been a pragmatic clinical goal.

Rhythmic production of insulin regulated by peripheral β-cell clocks was revealed by continuous perifusion of isolated islets from the rat, which has a 24-hour rhythm (*49*). Live-cell imaging in islets isolated from *Per2-Luciferase* mice shows that they express a self-sustained oscillator with period length matching that of the liver and pituitary (*50*). $Clock^{\Delta 19}$ mutant mice develop age-dependent hyperglycemia in both the light and dark phases of the cycle, corresponding with periods of fasting and feeding (*29*). These animals also develop susceptibility to diet-induced obesity; however, rather than displaying the anticipated hyperinsulinemia, they instead have inappropriately low concentrations of insulin. $Clock^{\Delta 19}$ mutant mice display a steeper drop in blood sugar in response to treatment with insulin, a sign that these animals have enhanced insulin sensitivity, thereby masking their β-cell deficiency (*50*). *Bmal1* mutant mice also have impaired glucose tolerance (*51*), increased insulin sensitivity, and a progressive myopathy with aging that causes cachexia, which limits interpretation of glucose turnover studies. Studies in isolated islets revealed first that the clock oscillator is expressed and self-sustained in this tissue and, second, that glucose responsiveness in islets is diminished when the clock is disrupted. After middle age, the mutants also have smaller islet size, reduced proliferation, and transcriptome-wide decreases in proliferative gene expression. Studies in tissue-specific knockout mice have supported the hypothesis that function of the clock activators in the liver opposes their function in the pancreas (*51*). Whereas ablation of *Bmal1* exclusively within the islet does not affect activity behavior, feeding, or body weight, these mice display a much

greater impairment of glucose tolerance than the global knockout, as predicted. Islets from both global and pancreas-specific knockouts have normal insulin content, and influx of calcium in response to glucose is intact. However, exocytosis is impaired, suggesting that the clock controls the latest stage in stimulus-secretion coupling.

Findings in experimental genetic models of clock-gene ablation may also have implications for understanding emerging evidence that the circadian system participates in human glucose metabolism. For instance, in genome-wide association studies, variation in the *Melatonin 1b receptor* (*MTNR1B*) and in *Cry2* are both associated with blood glucose concentrations [(*52*) and reviewed in (*53*)]. MTNR1B, the cognate receptor of the circadian-regulated hormone melatonin, is expressed in many metabolic tissues, whereas *Cry2* encodes a clock repressor. These findings underscore the need to incorporate temporal considerations at the planning stages in future studies to account for circadian variation. Similarly, temporal considerations may aid in analysis of experimental genetic models because testing at different times and under different environmental light cycles may uncover unanticipated effects.

Sleep and forced circadian misalignment: genetic models and human studies. Ties between circadian disruption and metabolic disturbance have garnered attention, including large cross-sectional sampling of populations subjected to shift work. Extensive studies also indicate a correlation between sleep time and body mass index (BMI). Disruption in specific phases of sleep may be connected to metabolic function. Subtle tones sufficient to selectively deprive subjects of slow-wave sleep without producing conscious wakefulness were sufficient to impair glucose tolerance (*54*). Neuroanatomic studies also indicate interconnections between regions of hypothalamus important in circadian signaling, energetics, and sleep (*55*, *56*). At the molecular level, orexin (also termed hypocretin), originally discovered as a neuropeptide produced in the feeding-stimulatory neurons of lateral hypothalamus, is positioned at the intersection of neuronal systems controlling sleep, circadian output, and metabolism (*56*). Analysis of orexin receptor 2 knockout mice indicates that lack of orexin signaling increases susceptibility to obesity (rather than the original expectation that orexin, a potent wakefulness-inducing peptide, would induce adiposity) (*57*). Orexin receptor 2 mutations also account for canine narcolepsy, and orexin deficiency is a hallmark of the disease in humans (*58*). Activity of the orexin neuron is modulated by glucose and integrates signals downstream of leptin-responsive neurons within the arcuate nucleus. Leptin also affects sleep, possibly independently of effects on body weight, raising the need to further define leptin actions in this process (*59*). Manipulation of orexin signaling, an integrator of energetic and circadian signals, may thus provide opportunities to intervene not only in disorders of sleep but also in related metabolic complications.

In humans exposed to a light/dark cycle lengthened to 28 hours, out of synchrony with the endogenous clock, the sleep/wake cycle is driven at 28 hours, whereas the melatonin and body-temperature rhythm free-runs with a ~24-hour period (*60*). Such "forced desynchrony," a manipulation that is intended to simulate deleterious effects of jet lag or shift work, caused impaired glucose tolerance and hypoleptinemia. Whether circadian disruption might also affect endocrine pancreas insulin secretion, hepatic gluconeogenesis, and glucose disposal in skeletal muscle in humans awaits further study; however, these results emphasize the clinical linkages between circadian function and metabolic homeostasis.

Coupling and Outputs: How Do Clocks Sense and Respond to Nutrient Signals?

Under homeostatic conditions, the clock acts as a driver of metabolic physiology (Fig. 3). However, with perturbations in either circadian or metabolic systems, such as forced behavioral misalignment with shift work or, conversely, high-fat feeding, a vicious cycle ensues in which disruption of metabolic pathways damp and lengthen circadian oscillations (*61*). The identity of metabolic sensors that may act as intermediates in coupling circadian cycles with physiologic systems remains to be identified. For instance, do changes in cell-

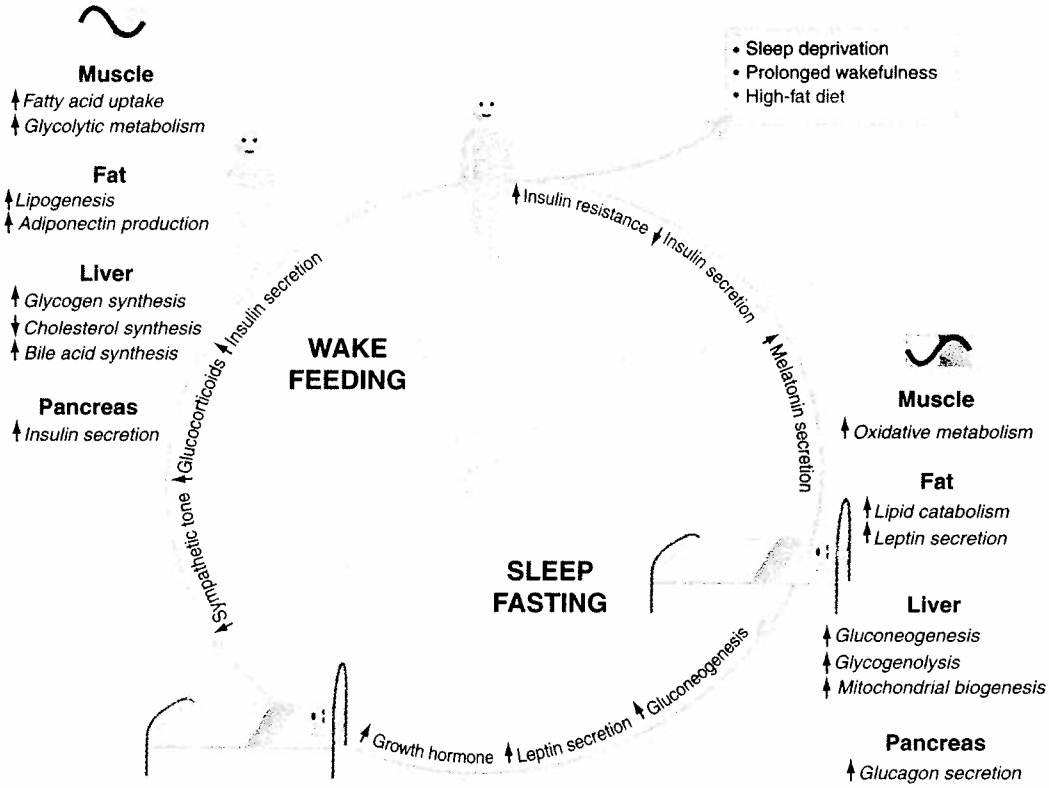

Fig. 3. The clock partitions behavioral and metabolic processes according to time of day. The clock coordinates appropriate metabolic responses within peripheral tissues with the light/dark cycle. For example, the liver clock promotes gluconeogenesis and glycogenolysis during the sleep/fasting period, whereas it promotes glycogen and cholesterol synthesis during the wake/feeding period. Proper functioning of peripheral clocks keeps metabolic processes in synchrony with the environment, which is critical for maintaining health of the organism. Different tissues exhibit distinct clock-controlled properties; thus, ablation of the clock in certain tissues will cause opposing effects on metabolic function as uncovered through dynamic challenges at different times in the cycle under different nutrient conditions. Aging, diet, and environmental disruption such as shift work may also affect the integration of circadian and metabolic systems.

nutrient signaling in turn produce changes in circadian clock function? Does metabolic disease lead to altered amplitude or phase of circadian cycles within brain or peripheral organs? Two lines of research have begun to address these questions: first, involving the cellular pathway of AMP concentrations, and second, involving NAD^+ metabolism. Using phosphopeptide mapping, Lamia *et al.* identified a consensus motif for phosphorylation by AMP-activated protein kinase (AMPK), a sensor of AMP/ATP (adenosine triphosphate) ratio, within the CRY protein (*62*). AMP kinase activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) promoted degradation of CRY, which was abrogated by mutation of the AMPK consensus motif. AMPK knockout mouse embryonic fibroblasts (MEFs) also displayed altered rhythmicity, leading to the proposal that AMP concentration directly couples circadian rhythms to nutrient state in peripheral cells.

A second example in metabolic coupling with the core clock originated with the finding that the mammalian ortholog of the yeast sirtuin deacetylases (for silent information regulator)—proteins that activate or silence chromatin according to availability of fuel—comprises part of an additional feedback loop with the core clock (*63*, *64*). Sirtuins are present in transcription complexes with the clock and in turn modulate activity of clock transcription factors. CLOCK-BMAL1 activates the major pathway for mammalian NAD^+ synthesis, involving its regeneration from nicotinamide mediated by nicotinamide phosphoribosyltransferase (NAMPT) (*65*) (*66*). NAD^+ concentration in cells varies across the light/dark cycle, consistent with a role for NAD^+ as an oscillating metabolite linking metabolic cycles with the clock. Both NAMPT and SIRT1, similar to the NHRs and AMPK, are regulated not only by the clock but also by the nutritional status of the organism. For example, fasting increases NAMPT expression in an AMPK-dependent manner in skeletal muscle, whereas fasting and caloric restriction increase SIRT1 activity across multiple tissues. Thus, regulation of the clock by NAD^+ and SIRT1 allows for fine-tuning and synchronization of the core molecular clock with the environment. Because NAD^+-dependent deacetylases regulate gluconeogenesis and many other pathways (*67*), it will be important to further delineate the role of the clock in NAD^+-driven metabolism (Fig. 2). A second NAD^+-regulated pathway has recently been linked to circadian feeding cycles: PARP-1 activity is circadian in the liver, causing the rhythmic addition of poly-adenosine-diphosphate-ribose residues to the CLOCK protein. Because PARP-1 is regulated by NAD^+, this provides yet another pathway for metabolic signals to regulate the core clock pathway (*68*). Collectively, these findings identify incoming (AMPK and PARP-1) and outgoing (NAD^+/Sirtuin) sensors that couple nutrient availability, metabolism, and the clock.

Conclusion

In just the past 20 years, the mystery of biological timing has been transformed through genetic discovery. As a consequence, the availability of molecular clock genes has now provided tools to understand the physiological functions of the circadian system in unprecedented detail. As we experimentally dismantle the clock, the interdependence of timing and energetics seems inextricable. Major gaps in our understanding include (i) the connection between brain and peripheral tissue clocks in metabolic homeostasis, (ii) the interplay between circadian and sleep disruption in energetics, (iii) the relationship between nutrient state and circadian homeostasis, and (iv) the impact of circadian clock systems on human physiology. Ultimately, such studies will yield deeper insight into the interconnections between genes, behavior, and metabolic disease.

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REVIEW

Manufacturing Molecules Through Metabolic Engineering

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Metabolic engineering has the potential to produce from simple, readily available, inexpensive starting materials a large number of chemicals that are currently derived from nonrenewable resources or limited natural resources. Microbial production of natural products has been achieved by transferring product-specific enzymes or entire metabolic pathways from rare or genetically intractable organisms to those that can be readily engineered, and production of unnatural specialty chemicals, bulk chemicals, and fuels has been enabled by combining enzymes or pathways from different hosts into a single microorganism and by engineering enzymes to have new function. Whereas existing production routes use well-known, safe, industrial microorganisms, future production schemes may include designer cells that are tailor-made for the desired chemical and production process. In any future, metabolic engineering will soon rival and potentially eclipse synthetic organic chemistry.

The term "metabolic engineering" was coined in the late 1980s–early 1990s (*1*). Since that time, the range of chemicals that can be produced has expanded substantially, in part due to notable advances in fields adjacent to metabolic engineering: DNA sequencing efforts have revealed new metabolic reactions and variants of enzymes from many different organisms; extensive databases of gene expression, metabolic reactions, and enzyme structures allow one to query for desired reactions and design or evolve novel enzymes for reactions that do not exist; new genetic tools enable more precise control over metabolic pathways; new analytical tools enable the metabolic engineer to track RNA, protein, and metabolites in a cell to identify pathway bottlenecks; and detailed models of biology aid in the design of enzymes and metabolic pathways. Yet even with these substantial developments, microbial catalysts are not as malleable as those in synthetic organic chemistry, and metabolic engineers must weigh many trade-offs in the development of microbial catalysts: (i) cost and availability of starting materials (e.g., carbon substrates); (ii) metabolic route and corresponding genes encoding the enzymes in the pathway to produce the desired product; (iii) most appropriate microbial host; (iv) robust and responsive genetic control system for the desired pathways and chosen host; (v) methods for debugging and debottlenecking the constructed pathway; and (vi) ways to maximize yields, titers, and productivities (Fig. 1). Unfortunately, these design decisions cannot be made independently of each other: Genes cannot be expressed, nor will the resulting enzymes function, in every host; products or metabolic intermediates may be toxic to one host but not another host; different hosts have different levels of sophistication of genetic tools available; and processing conditions (e.g., growth, production, product separation and purification) are not compatible with all hosts. Even with these many challenges, metabolic engineering has been successful for many applications, and with continued developments more applications will be possible.

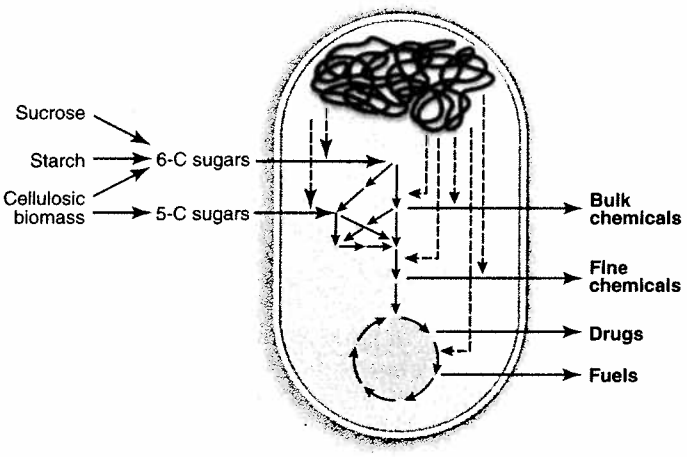

Fig. 1. Conversion of sugars to chemicals by means of microbial catalysts.

Starting Materials, Products, and Metabolic Routes

One area where metabolic engineering has a sizable advantage over synthetic organic chemistry is in the production of natural products, particularly active pharmaceutical ingredients (APIs), some of which are too complex to be chemically synthesized and yet have a value that justifies the cost of developing a genetically engineered microorganism. The cost of starting materials is generally a small fraction of their cost, and relatively little starting material is necessary so availability is not an issue. Most APIs fall into three classes of natural products, and many of the biosynthetic pathways for their precursors have been reconstituted in heterologous hosts.

Alkaloids are nitrogen-containing, low molecular weight compounds found primarily in and derived from plants and widely used as drugs. Two recent studies conclude that the large group of benzyl isoquinoline alkaloids (BIAs) will one day be producible in *Escherichia coli* and *Saccharomyces cerevisiae* (*2*). Unfortunately, the BIAs are only one of four major alkaloid groups, all of which are produced through different pathways. As the metabolic pathways for other alkaloids are discovered in their natural producers, many more of these valuable molecules could be produced microbially.

Polyketides and nonribosomal peptides (NRPs) have found broad use as APIs, veterinary agents, and agrochemicals. Naturally occurring polyketides and NRPs are produced by a number of bacteria and fungi using large, modular enzymes. Their titers and yields in the native producers have been improved through traditional strain engineering and advanced metabolic engineering. More recently, some of the most valuable molecules have been produced with engineered industrial hosts (*3*). Recombination of various synthase modules allows one to produce a nearly infinite range of chemicals (*4*, *5*), opening up the possibility that they may one day be used to produce fine and bulk chemicals.

Isoprenoids have found use as fragrances and essential oils, nutraceuticals, and pharmaceuticals. Many isoprenoids have been produced microbially, including carotenoids and various plant-derived terpenes (*6*–*8*), taking advantage of terpene synthases to form the most complicated part of the molecules and hydroxylases to introduce hydroxyl group that can be subsequently functionalized chemically or biologically (*7*, *9*). Isoprenoids are one of the few classes of natural products where there are alternative precursor production pathways. An example of using metabolic engineering and synthetic chemistry together to produce an API is the semisynthesis of the antimalarial drug artemisinin with *S. cerevisiae* engineered to produce artemisinic acid, the most complex part of the molecule, and synthetic chemistry to produce artemisinin from the microbially sourced

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artemisinic acid (*6*, *7*, *9*). Beyond producing natural products, laboratory evolution or rational engineering of terpene cyclases, terpene hydroxylases, and a host of other terpene-functionalizing enzymes (*8*, *10*–*12*) and combinatorial expression of these evolved enzymes in a heterologous host will enable the production of unnatural terpenes, some of which might be more effective than the natural product for the treatment of human disease.

Although individual metabolic pathways have been developed to produce natural products derived from a single pathway, there is an opportunity to synthesize multisubstituent APIs (e.g., Taxol) or other molecules from the products of multiple biosynthetic pathways. This will require simultaneous expression of multiple precursor pathways in a single microorganism, as well as "ligases" that can assemble multiple substituents together into a single molecule. The benefit would be the synthesis of complicated molecules that might not otherwise be produced.

Although not as valuable as pharmaceuticals, many fine chemicals have been produced economically from natural and engineered microorganisms, including amino acids, organic acids, vitamins, flavors, fragrances, and nutraceuticals. For fine chemicals, profit margins are generally much lower than for APIs and may be affected by substrate availability and cost. Some of these molecules are sufficiently complicated that they cannot be produced economically by any route other than biological production, whereas others have chemical routes. For some important products (fragrances, flavors, amino acids), heterologous hosts have been engineered to enhance their production. Yet we have barely begun to investigate what will be possible to produce.

In contrast, bulk chemicals such as solvents and polymer precursors are rarely produced from microorganisms, because they can be produced inexpensively from petroleum by chemical catalysis. Due to fluctuations in petroleum prices and recognition of dwindling reserves, trade imbalances, and political considerations, it is now possible to consider production of these inexpensive chemicals from low-cost starting materials such as starch, sucrose, or cellulosic biomass (e.g., agricultural and forest waste, dedicated energy crops, etc.) with a microbial catalyst. For example, 1,3-propanediol (1,3-PDE), a useful intermediate in the synthesis of polyurethanes and polyesters, is now being produced from glucose by *E. coli* engineered with genes from *Klebsiella pneumoniae* and *S. cerevisiae* (*13*). There is an opportunity to produce many other bulk chemicals (e.g., polymer precursors) by using metabolically engineered cells, but the key will be to produce the exact molecule needed for existing products rather than something "similar but green" that will require extensive product testing before it can be used.

By far the highest-volume (and lowest-margin) application for engineered metabolism is the production of transportation fuels. For many of the same reasons that it is desirable to produce petroleum-derived chemicals using biological systems, it is desirable to produce transportation fuels from readily available, inexpensive, renewable sources of carbon. There is a long history of using microorganisms to produce alcohols, primarily ethanol and butanol. Although much of the work on these alcohols was done by traditional strain mutagenesis and selection, more recent work focused on engineering yeasts and bacteria to produce ethanol or butanol from a variety of sugars while eliminating routes to side products and improving the tolerance of the host to the alcohol (*14*). Larger, branched-chain alcohols can be produced by way of the Ehrlich pathway. By incorporating broad substrate-range 2-keto acid decarboxylases and alcohol dehydrogenases, several microbes have now been engineered to produce these fuels (*15*, *16*). These alcohols are generally considered better fuels than ethanol and butanol and can also be used to produce a variety of commodity chemicals.

Recent advances in metabolic pathway and protein engineering have made it possible to engineer microorganisms to produce hydrocarbons with properties similar or identical to those of petroleum-derived fuels and thus compatible with our existing transportation infrastructure. Linear hydrocarbons (alkanes, alkenes, and esters) typical of diesel and jet fuel can be produced by way of the fatty acid biosynthetic pathway (*17*–*19*). For diesel in cold weather and jet fuel at high altitudes, branches in the chain are beneficial—regularly branched and cyclic hydrocarbons of different sizes with diverse structural and chemical properties can be produced via the isoprenoid biosynthetic pathway (*20*, *21*). Both the fatty acid–derived and the isoprenoid-derived fuels diffuse (or are pumped) out of the engineered cells and phase separate in the fermentation, making purification simple and reducing fuel cost.

Although the pathways described above produce a wide range of fuel-like molecules, there are many other molecules that one might want to produce, such as short, highly branched hydrocarbons (e.g., 2,2,4-trimethyl pentane or isooctane) that would be excellent substitutes for petroleum-derived gasoline. Additionally, most petroleum fuels are mixtures of large numbers of components that together create the many important properties of the fuels. It should be possible to engineer single microbes or microbial consortia to produce a mixture of fuels from one of the biosynthetic pathways or from multiple biosynthetic pathways. Indeed, some enzymes produce mixtures of products from a single precursor—maybe these enzymes could be tuned to produce a fuel mixture ideal for a particular engine type or climate.

To make these new fuels economically viable, we must tap into inexpensive carbon sources (namely, sugars from cellulosic biomass). Given the variety of sugars in cellulosic biomass, the fuel producer must be able to consume both five- and six-carbon sugars. Because many yeasts do not consume five-carbon sugars, recent developments in engineering yeast to catabolize these sugars will make production of these fuels more economically viable (*22*). Engineering fuel-producing microorganisms to secrete cellulases and hemicellulases to depolymerize these sugar polymers into sugars before uptake and conversion into fuels has the potential to substantially reduce the cost of producing the fuel.

Hosts and Expression Systems

From the applications cited above, it should be evident that the product, starting materials, and production process all affect host choice. Some of the most important qualities one must consider when choosing a host are whether the desired metabolic pathway exists or can be reconstituted in that host; if the host can survive (and thrive) under the desired process conditions (e.g., ambient versus extremes of temperature, pH, ionic strength, etc.); if the host is genetically stable (both with the introduced pathway and not susceptible to phage attack); and if good genetic tools are available to

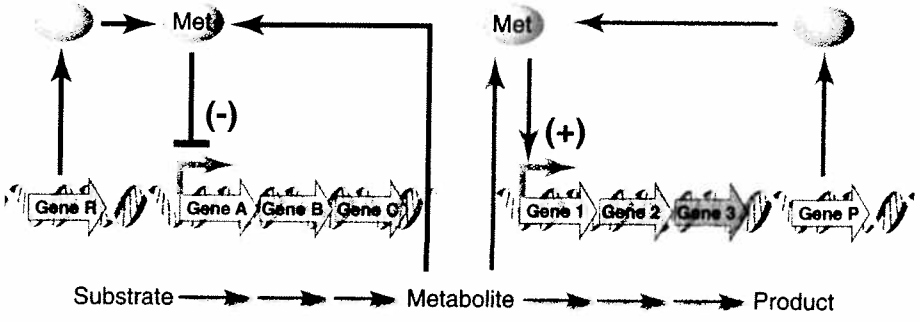

Fig. 2. Use of synthetic regulators to modulate metabolic pathways that have a toxic intermediate. Regulatory proteins or RNAs bind the toxic metabolite and down-regulate the biosynthetic pathway and up-regulate the consumption pathway.

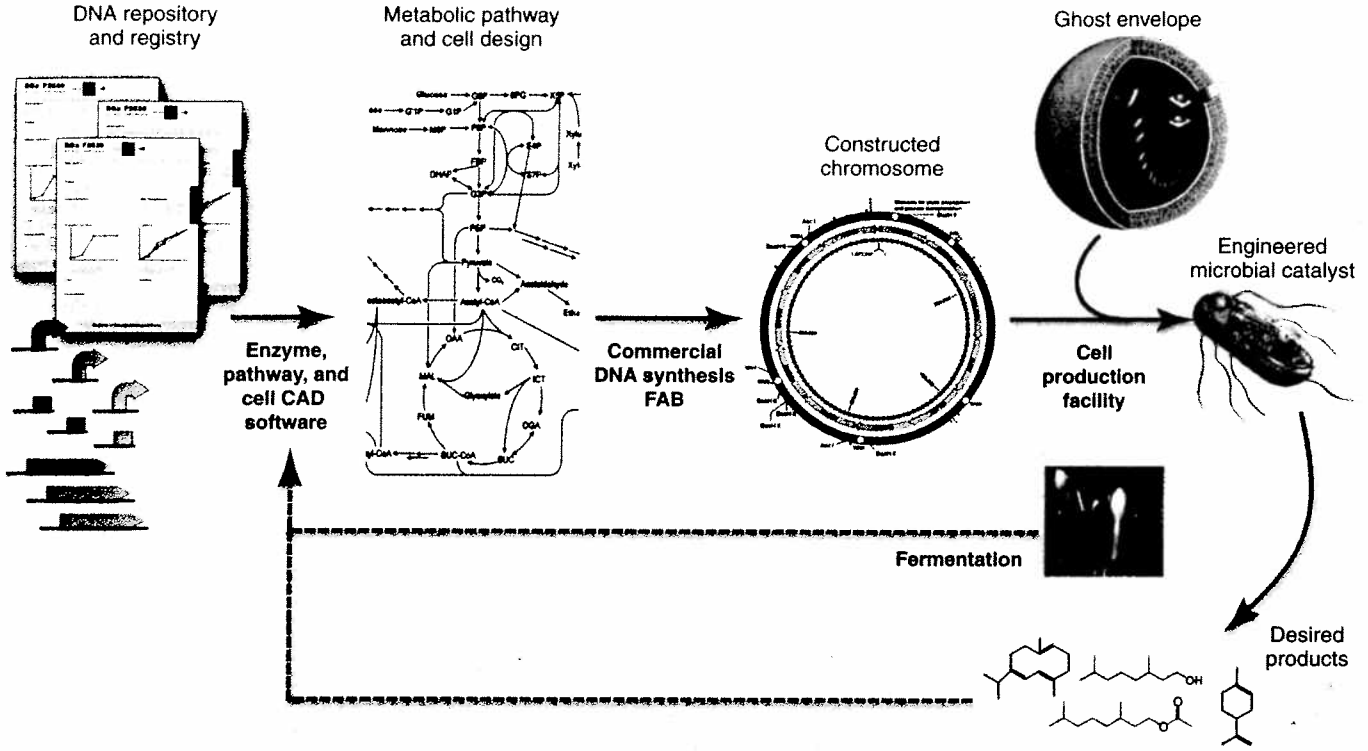

Fig. 3. The future of engineered biocatalysts. Pathways, enzymes, and genetic controls are designed from characteristics of parts (enzymes, promoters, etc.) by means of pathway and enzyme CAD software. The chromosomes encoding those elements are synthesized at a FAB and incorporated into a ghost envelope to obtain the new catalyst. The design of the engineered catalyst is influenced by the desired product and the production process.

manipulate the host. Widely used, heterologous hosts include *E. coli*, *S. cerevisiae*, *Bacillus subtilis*, and *Streptomyces coelicolor*, to name a few. Although *E. coli* and *S. cerevisiae* excel in the genetic tools available, *E. coli* has the disadvantage of being susceptible to phage attack. And while *B. subtilis* and *S. coelicolor* have the ability to easily express polyketide synthases, they have fewer genetic tools available than either *S. cerevisiae* or *E. coli*. Although minimal, bacterial hosts may have scientific interest (*23*), minimal hosts that require addition of many nutrients or that cannot cope with stresses in processing will probably not find a niche in industrial chemical or fuel production where cost is critical. Thus, it is essential to have genetic tools for existing industrial hosts that can grow on simple, inexpensive carbon sources and salts or on an inexpensive, undefined medium with minimal additions (*24*, *25*).

The key issue necessitating good genetic tools is the introduction of foreign genes encoding the metabolic pathway and control over their expression to maximize yields and titers. The genes encoding the transformational enzymes in metabolically engineered cells do not need to be highly expressed, but must be produced in catalytic amounts sufficient to adequately transform the metabolic intermediates into the desired products at a sufficient rate. Expression of the desired genes at too high a level will rob the cell of metabolites that might otherwise be used to produce the desired molecule of interest, particularly important for production of low-margin chemicals, while underexpressed genes will create pathway bottlenecks. Furthermore, because intermediates of a foreign metabolic pathway can be toxic to the heterologous host (*6*), which results in decreased production of the desired final compound, it is essential that the relative levels of the enzymes be coordinated.

Central to any genetic manipulation is the vector used to carry and/or harbor the transforming DNA in the host. Important features of the cloning vector include segregational stability, minimal and consistent copy number in all cells of a culture, and the ability to replicate and express large sequences of DNA. There is growing recognition that one or only a few copies of a gene are needed, particularly for metabolic engineering applications. With the ability to vary promoter (*26*) and ribosome binding strength (*27*), as well as the stabilities of the mRNA (*28*) and the resulting protein produced from it, there are many factors other than copy number that can be manipulated to alter enzyme production.

Promoters play an essential role in controlling biosynthetic pathways. Inducible promoters are one of the easiest and most effective ways to regulate gene expression, but it is essential that the promoter be induced consistently in all cells of a culture (*29*). Constitutive promoters (*26*) and promoters that respond to a change in growth condition or to an important intermediary metabolite (*30*) allow for inexpensive, inducer-free gene expression, which is particularly important where cost is an issue (Fig. 2). Although there are many inducible promoters for bacteria, the small number of inducible promoters for yeast and other potential industrial hosts makes regulation of metabolic pathways in those organisms more challenging than in bacteria.

Because production of complicated molecules often requires several enzymes, it is desirable to coordinate expression of the genes encoding these enzymes to prevent accumulation of toxic intermediates and bottlenecks in biosynthetic pathways. There are many ways to coordinate expression of multiple genes, such as using a nonnative RNA polymerase or transcription factor to induce multiple promoters (*31*); grouping multiple, related genes into operons; varying the ribosome binding strength for the enzymes encoded in the operon (*27*); or controlling segmental mRNA stability of each coding region to regulate the amount of each enzyme produced (*32*). One of the limitations to expressing multiple genes in yeast is the lack of internal ribosomal entry sequences (IRESs) that are available for higher eukaryotes. The development of yeast IRESs would allow one to express genes encoding metabolic

pathways without the need for a promoter for each gene.

Debottlenecking, Debugging, and Process Optimizing

Even with a kit full of tools, building a biosynthetic pathway is made difficult without accurate blueprints. In almost all areas of engineering, there are models and simulation tools that allow one to predict which components to assemble to obtain a larger system with a desired function or characteristic. Similar biological design tools are in their infancy. However, metabolic models that incorporate cell composition and gene regulation have become relatively predictive and may one day be used to design metabolic pathways and predict the level of gene expression needed to achieve a particular flux through a reaction or pathway (*33*).

Regardless of how sophisticated the design tools and how good the blueprint, there will always be "bugs" in the engineered system. Analogous to software debuggers that allow one to find and fix errors in computer code, the development of similar tools for biological debugging would reduce development times for optimizing engineered cells. For the development of microbial chemical factories, functional genomics can serve in the role of debugging routines (*34*), because imbalances in a metabolic pathway often elicit a stress response in central metabolism (due to protein overproduction or accumulation of toxic intermediates or end products) (*6*, *35*). Information from one or more of these techniques can be used to diagnose the problem and modify expression of genes in the metabolic pathway or in the host to improve titer and/or productivity.

Many desirable chemicals will be toxic to the producer, particularly at the high titers needed for industrial-scale production. Taking advantage of the cell's native stress response pathways can be an effective way to alleviate part or all of the toxicity (*36*). Even better, transporters could be used to pump the desired product outside the cell, reducing intracellular toxicity and purifying the product from the thousands of contaminating intracellular metabolites (*37*).

Designer Cells for Designer Chemicals

One can envision a future when a microorganism is tailor-made for production of a specific chemical from a specific starting material, much like chemical engineers build refineries and other chemical factories from unit operations (Fig. 3). The chemical and physical characteristics of the product and starting materials would be considered in the design of the organism to minimize both production and purification costs (e.g., operating the engineered cell at the boiling point of volatile, toxic products to drive production and reduce product toxicity). The cell envelope would be designed to be resistant to the specific desired chemical, and the cell wall would be designed to make the organism tolerant to industrial processing conditions. Specific, engineered transporters would be incorporated into the membrane to pump the desired product out of the cell and keep it out and to import the desired starting material. The biosynthetic pathway would be constructed from a parts registry containing all known enzymes by means of retrosynthesis software (*38*), and done so to maximize yield and minimize the time required to grow the organism and produce the desired chemical from the desired starting material. In the event that an enzyme does not exist for a particular reaction or set of reactions, one would use computer-aided design (CAD) software to design the desired enzyme (*39*).

Once the cell has been designed in the computer, the genetic control system would be designed to control expression of all the genes at the correct time and at the appropriate levels. Redundancies in the genetic control system would be engineered to ensure that design parameters are maintained regardless of the transient changes the cell encounters during the production process. Simulations and scenario planning would test various designs, including genetic control system failure. Safety for the plant operators and the environment would be an essential design criterion. When the genetic controls were fully designed and tested, the chromosome(s) would be designed and constructed. Gene location, modularity, and ease of construction are but a few of the important considerations in designing the chromosome. The chromosome would be ordered from a commercial DNA manufacturer. Depending on the state of the technology at the time, the chromosome would arrive in pieces and be assembled in the constructed envelope or would be completely assembled at the factory and sent to another location to be introduced into the ghost cell. One can even envision a day when cell manufacturing is done by different companies, each specializing in certain aspects of the synthesis—one company constructs the chromosome, one company builds the membrane and cell wall (the "bag"), one company fills the bag with the basic molecules needed to boot up the cell.

Until this future arrives, manufacturing of molecules will be done with well-known, safe, industrial microorganisms that have tractable genetic systems. Continued development of tools for existing, safe, industrial hosts, cloning and expressing genes encoding precursor production pathways, and the creation of novel enzymes that catalyze unnatural reactions will be necessary to expand the range of products that can be produced from biological systems. When more of these tools are available, metabolic engineering should be just as powerful as synthetic chemistry, and together the two disciplines can greatly expand the number of products available from renewable resources.

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