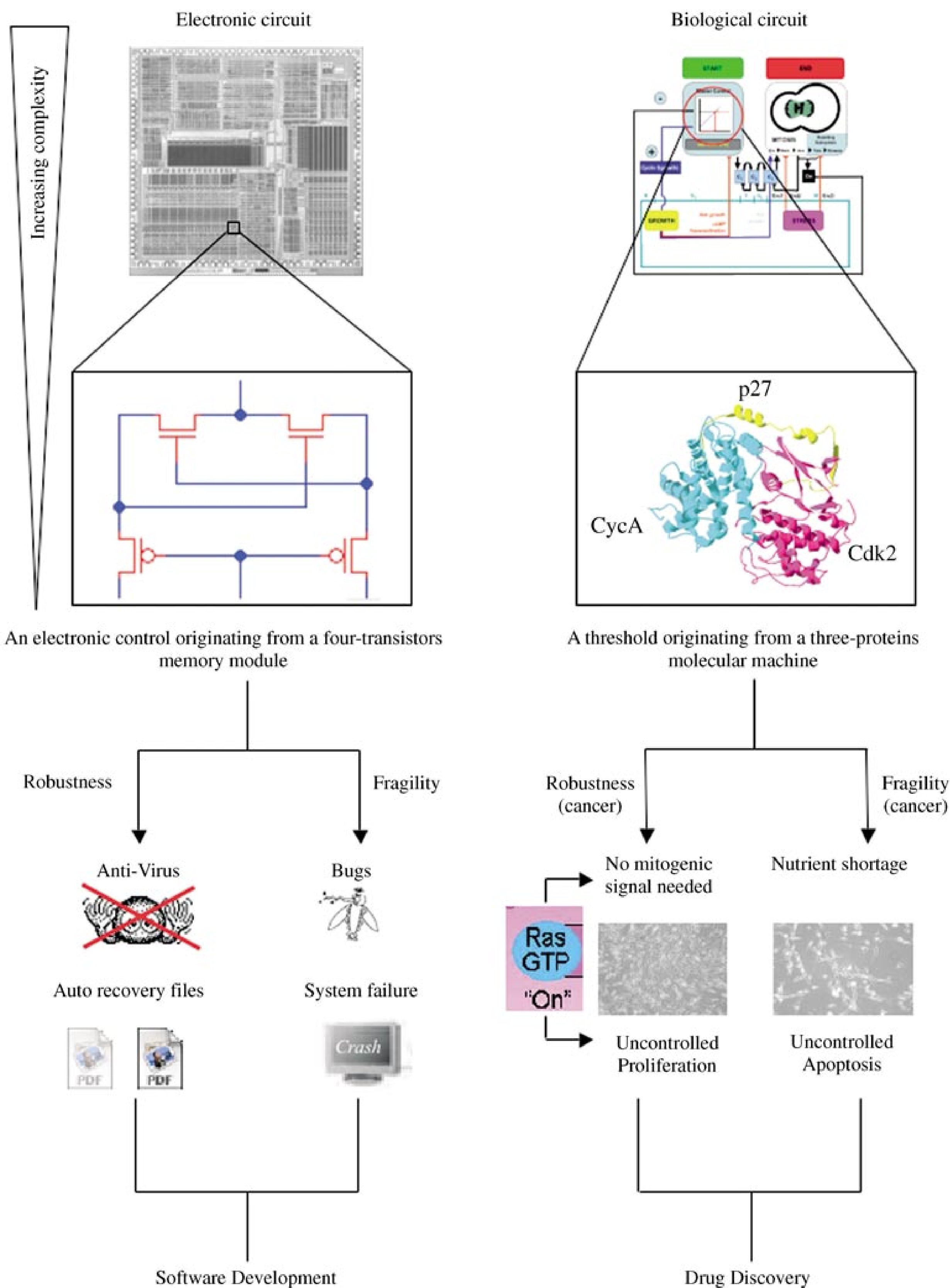

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Systems Biology and the Molecular Circuits of Cancer

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Proliferative disorders are a major challenge for human health. The understanding of the organization of cell-cycle events is of the utmost importance to devise effective therapeutic strategies for cancer. The awareness that cells and organisms are complex, modular, hierarchical systems and the availability of genome-wide gene expression and protein analyses, should make it feasible to elucidate human diseases in terms of dysfunctions of molecular systems. Here we review evidence in support of a systems model of the cell cycle, in which two sequential growth-sensitive thresholds control entry into S-phase. The putative molecular determinants that set the threshold for entry into S-phase are consistently altered in cancer cells. Such a framework could be useful in guiding both experimental investigation and data analysis by allowing wiring to other relevant cell modules thereby highlighting the differential responses, or lack of response of cancer cells to intra- and extracellular factors. Pharmacological approaches that take advantage of transformation-induced fragility to glucose shortage are discussed. Extension of this hierarchical, modular approach to tumors as a whole holds promise for the development of effective drug discovery approaches and more efficient therapeutic protocols.

Introduction

Cell proliferation requires coordination and integration of different processes to modulate the activity of key cell-cycle regulators. They are controlled by numerous mechanisms that reflect the diversity of the signals they integrate and the central importance of their role in cell-cycle control. Proliferative disorders are a major challenge for human health, uncontrolled cell proliferation being the hallmark of cancer. In fact, in tumor cells, the balance between intra- and extracellular signals and the control of the cell cycle is lost. To understand how intracellular and extracellular signals are transmitted to the cell-cycle machinery and how the latter adjusts its frequencies accordingly is one of the major challenges in molecular biomedicine. For many years research into the molecular basis of diseases focused on the products of individual genes. These were examined in parallel by different groups. Rarely were they studied in terms of the complete intracellular networks they are a part of. Neither the proper tools nor the data for such a network-based analysis were available. Indeed only recently has it become possible to analyze the expression of all genes in a pathway simultaneously. Furthermore, the genomics revolution has opened the way to other similar global approaches, such as proteomics and metabolomics. Considering the large amount of data emerging from these high-throughput techniques, only the development of new computer sciences and modeling methodologies will enable us to select the relevant from the irrelevant information and utilize it for health-care applications. In fact, molecular biology should begin to address the organization of the large network of molecules that determine cellular functions and their disturbance. In other words, molecular medicine has to be understood in terms of the functioning of modular *networks of molecules*. The result of this integrative/interactive process is very relevant for two reasons. First, it will make it possible to analyze and understand the molecular basis of each disease. Thanks to the completeness of the information (genomics, proteomics, and metabolomics), all molecular determinants can in principle be identified, produced, and analyzed in terms of structure and manipulated to elucidate their function. Second, most diseases are multifactorial in their pathology, if not in their origin. Accordingly, understanding disease and the design of personalized therapy requires going beyond the product of a single gene, to all gene products with which that gene interacts, if not to the functioning of the entire system of interacting molecules. Furthermore, the interactive process of model construction and validation will enable identification of the robustness and fragility of regulatory molecular circuits that are altered in a given pathological state. Thereby a strategy for the rational selection of specific molecular targets for drug discovery and development can be developed.

Systems Biology: Towards an Integrated Understanding of Biology in the Post-Genomic Era

Multicellular organisms' functions are regulated through a limited number of interactions among a limited number of organs, each formed by a restricted number of cell types. These interactions are modulated by environmental, intra- and intercellular signals. On the other hand each eukaryotic cell is very complex since it is composed of an exceedingly large number of differently active macromolecules that interact with each other and with low molecular weight components to

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Ferdinando Chiaradonna, was born in Napoli, Italy. He studied Biology at the University of Napoli where he graduated in 1993. In the same year he moved to the International Institute of Genetics and Biophysics in Naples, where he worked on the characterization of urokinase in signal transduction. In 1996, he worked at EMBL in Heidelberg. In 1997 he moved to the European Institute of Oncology in Milano, where he started his PhD, working on identifying transcriptional targets of fusion proteins involved in the onset of leukaemia. Since 2002, he is Assistant Professor at the University of Milano-Bicocca. His research interests are currently focused on the molecular and systems-level investigation of neoplastic transformation induced by activation of the Ras pathway.

yield nonlinear behavior that has been fine tuned by natural selection to achieve specific functional properties. Furthermore, cellular processes may be disassembled into basic "operating units" or "modules", subsystems of interacting molecules (protein, DNA, RNA and low molecular weight components) that perform a given function (for instance signal transduction, protein synthesis, cell-cycle regulation) in a way largely independent from the context.[1] Biological systems are therefore complex, but modular and hierarchical and this awareness opens new ways to biological understanding.

Genome-wide gene expression and protein analyses are establishing new powerful tools for the study of complex biological phenomena.[2,3] By the integration of modular and post-genomic analyses there is hope to elucidate human diseases in terms of dysfunctions of molecular systems[4,5] and therefore to achieve more rational and specific treatments. To this end, both bioinformatics and systems biology approaches are needed. Bioinformatics can be defined as the computational ability to extract information from raw data, for example the ability to predict the 3D structure of a protein from its encoding DNA sequence or to cluster large number of data, such as those obtained by genome-wide transcriptional profiling. It mostly yields information on the core components of a cell or on their relative proportion, but it does not address the functional interactions that yield a cell's regulatory network. Systems biology, on the other hand, aims to identify regulatory circuits that underlie a given biological function (for example metabolism, cell cycle, signal transduction, differentiation, etc.). It requires both quantitative knowledge of the components of the regulatory circuits and the map of their interactions. Mathematical models and computer simulations of the network allow the prediction of the dynamics of the system following genetic and/or environmental perturbations and studying emergent new properties, such as homeostasis and robustness, that cannot be derived directly from the reductionist characterization of individual components of the network.[6–9]

In conclusion, bioinformatics mining of "-omics" yields statistically relevant properties that are useful for systems biology from which iterative rounds of model building, prediction, experimentation, model refinement and development[6,10] are expected to give new strength and focus to hypothesis-driven research in the post-genomic era.

Systems biology may address biological processes the molecular components of which are well known (such as glycolysis) or the functions of which require the activity of a large number of components, many of which are not known, as is the case for cell cycle. In this latter instance, a complex process is conveniently disassembled into "modules".[1] A module is given, as outlined above, by any functional unit that performs a given task in a way largely independent from other modules within a cell. A module contains all interacting components required to perform a given function, acting as an insulated unit that may perform the same function over and over. The definition of what constitutes a module may not always be obvious, since in systems biology, a module not only has to be able to perform a given function, but must be able to respond appropriately to perturbations. So, for instance, in order to allow

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faithful reproduction of the regulation of the glucose flux in yeast cells, the glycolytic module has to be "extended" to accommodate glucose transport, NADH reoxidation and branches[11] (www.siliconcell.net). A property of cellular modules that is receiving increasing attention is robustness, a crucial property of living systems, which allows the maintenance of homeostasis. More formally, within a given module one or more key properties of a biochemical network are said to be robust when they are insensitive to the precise values of their biochemical parameters. A well-documented example is bacterial chemotaxis, where the precision of adaptation is robust and does not vary by perturbing the concentration of the major proteins of the network. In contrast, other properties of the same network, such as steady-state behavior and adaptation time, show strong variations in response to varying protein concentrations.[12]

Each cell can thus be viewed as a multimodule system, whose function depends on the ability to coordinately fine tune the function of each module and on the appropriate connections among modules. Each module interacts with other intracellular modules through exchange of material (i.e., metabolite(s)) and information (i.e., through intracellular messengers, regulatory interactions, protein–protein interactions, etc.). The properties of a module's components and molecular connections between them are analogous to the circuit diagram of an electrical device.

General design principles, derived from synthetic sciences and engineering, govern the interactions and the function of modules, that is, switch, threshold control, positive and negative feedback, amplification, robustness, and error correction.[1,5,13] It is interesting to point out that for systems analysis it is not necessary to know all the components of a module. Instead, the complete knowledge of a module's components is required if one wants to model the system following chemical kinetics (see for instance ref. [9]) or graph theory.[14] A given process can then be described by its blueprint, that is a map in which its basic modules and governing interactions are identified. The availability of such a blueprint allows the development of modeling programs in order to simulate how changes in the module structure or interactions affect the behavior of the system. The predictions could then be validated by comparison with experimental data. Once the modules and their wiring have been defined in a preliminary way, analysis moves to the identification of the molecular components of each module by data mining and experimental testing, so as to refine the blueprint down to the molecular level.[15]

Control of Cell Proliferation and Cancer

Most forms of cancer are multifactorial diseases. Stepwise mutations of multiple (proto)oncogenes are required to develop a transformed cell; fewer number of changes are required in rodent than in human cells (reviewed in ref. [16,17]). Despite the large number of molecular and morphological differences between normal and cancer cells, the aberrant growth of tumor cells is basically due to disruption of mechanisms that regulate cell cycle and cell survival.[18] Drugs that are used against cancer mostly act nonspecifically also in normal cells and their effectiveness is therefore reduced by their action on important normal tissue as well as by processes of detoxification, secretion, and repair by the tumor cells. Hence, the tumor as a whole acquires a "robust" phenotype that allows it to escape the host surveillance mechanisms and pharmacological therapies.[19] There is therefore a clear need to pinpoint specific molecular differences in basic control processes of proliferation and survival to develop a new class of more effective anticancer drugs.

Cell proliferation requires coordination of different processes: mass accumulation, DNA replication and cell division. This tight coordination allows the maintenance of cell size and faithful partitioning of genetic material and is based on the cell's ability to integrate external and metabolic signals with the activity of key cell-cycle regulators. They are controlled by numerous mechanisms reflecting the variety of signals that they are able to integrate and their central importance in cell-cycle control. In cancer cells the balance between intracellular and extracellular signals and the control of the cell cycle is disturbed.[20,21] To understand how intracellular and extracellular signals are received from the cell-cycle machinery and how it adjusts its timing accordingly, is one of the major challenges in molecular medicine.

Genome-wide approaches are focusing on the determination of gene expression by using DNA chips and related technologies.[22] Transcriptional profiles have identified molecular signatures for different types of tumors and they are used as diagnostic or prognostic tools to differentiate otherwise similar tumors that may differ in prognostic index and hence in therapeutic approach. Genome-wide data are thus used as a refinement of histological and immunohistological analyses that are used today in clinical practice (see for instance ref. [23,24]). It is likely that transcriptome or proteome data may tell us more about transformed phenotype when analyzed with a systems biology approach. In order to do so it would be useful to identify at least one meaningful cell-cycle control network and to compare the post-genomic analysis of normal and transformed cells with known control wiring.

As a first step in this effort the control network controlling entrance into S-phase in a well known model organism, the budding yeast *Saccharomyce cerevisiae*, has been investigated. The consistent evolutionary conservation of many gene products engaged in the execution and control of the cell cycle from yeast to mammalian cells[25,26] suggests that a similar conservation may be found for the core organization of the control circuits.

Cyclins, Cdks and Cki are the key components of the evolutionary conserved molecular machine driving the cell cycle

Cyclin-dependent kinases (Cdks) play an essential regulatory role in cell-cycle progression: it is in fact the sequential activation of Cdks by specific, unstable, regulatory subunits, named cyclins, that first triggers the onset of DNA replication and later initiates mitosis.[27,28] Cdk activity is tightly regulated by different molecular mechanisms[29] that include regulatory

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phosphorylations, differential expression and/or localization, and interaction with regulatory proteins, such as cyclin dependent kinase inhibitors (Cki) which inhibit Cdk activity by binding to Cyclin–Cdk complexes.

The evolutionary conservation of Cdks from yeast to mammalian cells is well established.[26,28] In budding yeast a single Cdk (Cdc28, now renamed Cdk1) is involved in the control of the cell cycle, while five Cdks active in the control of the cell cycle (out of a total of nine) have so far been identified in mammals. Cdk4, Cdk6 and Cdk2 are active during G1, Cdk2 during S-phase, Cdk1 during G2 and M, while Cdk7 is active during all cell-cycle phases. Recently the involvement of another Cdk, Cdk3, in the G0-G1 transition[30] has been reported. Each cyclin associates with one or two Cdks, and most cyclin-dependent kinases associate with one or two cyclins (reviewed in ref. [28]).

In the cell cycle, there are cyclins associated with G1 (Cln3 in budding yeast, cyclin D in mammals), S-phase (Clb5 and 6 in yeast, cyclin E and A in mammals), and mitosis (Clb1 and 2 in yeast, cyclin B and A in mammals). Both cyclins and Cdks have a wide degree of redundancy and it is currently believed that their specificity, which drives the cell cycle, is dependent more on their temporal expression and subcellular localization than on substrate specificity which is embedded in their molecular structure (reviewed in ref. [28]).

As the name implies, Ckis, regulate cell cycle by inhibiting Cdk activity. Nevertheless, in recent years it has become increasingly clear that many Ckis are multifunctional proteins (reviewed in ref. [31]). In budding yeast the Cki, Far1, affects cell-cycle progression and cytoskeletal organization as $p27^{Kip1}$[31–34] does in mammals. Cip/Kip family members stably associate with cyclin D1–Cdk4 to assemble them into higher order, enzymatically active complexes.[31–33] The Cip/Kip inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ share an homologous inhibitory domain.[35] The Kip/Cip proteins inhibit Cdk complexes containing cyclin D and E. Interestingly, as previously observed for Cdks and cyclins, also Cki function is evolutionarily conserved since a mammalian Cip/Kip protein can substitute for Sic1 in yeast and, conversely, Sic1 can inhibit cyclin A–Cdk2 complex in vitro.[36] Whether the yeast Sic1, Cki, has a scaffolding activity has not been directly addressed so far.

In mammals, beside the Kip/Cip group described above, the Ink4 proteins (p15, p16, p18, p14/p19) form a second Cki family that does not have homologues in yeast. The Cki, $p16^{Ink4}$ and $p15^{Ink4b}$, may inhibit the formation of the cyclin D–Cdk4 complex which is required with cyclin E–Cdk2 in late G1 to activate the transcriptional program that promotes passage to the S-phase.[37,38] $p19^{Arf}$ has been shown to activate the p53 transcription factor.

A threshold cell sizer involving Cln3 and Far1 controls entrance into S-phase in budding yeast

For many years it has been recognized that a critical cell size (called Ps) is required in yeast to enter S-phase.[39,40] This control is of utmost physiological relevance, since it allows the coordination of cell growth with cycle progression and is responsible for cell size homeostasis.[41] Recent work from our laboratory has allowed the identification of the molecular basis of the cell sizer in budding yeast. First of all, it has been recognized that the G1 cyclin, Cln3, is modulated by cell growth, its level being higher in fast than in slow growing cells.[42,43] Since the level of Cln3 is constant in G1 cells, its amount in the cell is proportional to cell mass. Increasing the level of Cln3, by over-expression or by a mutational stabilization of the protein, decreases both cell size and Ps, while in cells where the *cln3* gene has been disrupted, cell size and Ps increase (reviewed in ref. [44]). In cells undergoing a shift up from a poor to a rich medium the level of Cln3 increases since the cells are unable to enter S-phase.[45]

These data which are in apparent conflict can be settled if Cln3 is not the only determinant of the cell sizer, but if it acts together with an inhibitor of cyclin dependent kinase (Cki). A threshold given by an activator, that interacts with an inhibitor blocking its action (Figure 1 A), is a very simple and effective biochemical threshold mechanism.[1,13,46,47] The threshold is set by the level of the Cki (black line) received by new cells which remains approximately constant in G1 cells. The threshold is overcome when the activator cyclin subunit (blue line), the synthesis of which proceeds proportionally to cell mass, exceeds that of Cki. The threshold can be made irreversible by an ensuing Cki degradation. The response of the threshold is shown in red. The threshold value and hence its dynamics can be altered by changing the levels of the inhibitor (Figure 1 B), the activator (Figure 1 C) or both (Figure 1 D). Both an increase in cyclin and a decrease in Cki can accelerate overcoming the threshold. When both the cyclin activator and Cki inhibitor are up-regulated, different dynamics can result depending on the relative value assumed by each component. In the example reported in Figure 1 D, Cki increases more than the cyclin, so that overcoming the threshold takes place at a larger size than in the reference, "wild type" situation.

We have put forward the hypothesis that Far1, the Cki long known to inhibit the G1 to S transition in response to mating pheromones,[48] might also have a role in the mitotic cycle by cooperating with Cln3 in a nutritionally modulated threshold, which controls entry into the S-phase.[47] A basic blueprint of the cell cycle has been proposed based on a Cki–cyclin threshold acting as a START function in which a cell-sizer controls entrance into S-phase by activating waves of cyclins that set the timing for the onset of mitosis and cell division (Figure 2). Growth in rich media delays entrance into S-phase and stimulates the onset of mitosis.[42] Execution of mitotic events is inhibited by stress conditions (including mitotic spindle checkpoint, DNA damage,[49] hyperactivation of cAMP pathway in nitrogen poor media[50]). Computer simulation analyses based on an algorithm derived from the model in Figure 2 are able to predict with accuracy the dynamics of growth and budding in steady and transitory states.[47]

Direct molecular evidence giving strong support to the role of Far1 during the mitotic cycle has been recently obtained.[45] Overcoming the Cln3–Far1 threshold, is followed by a well known second threshold that requires the Cki, Sic1, and Clb5 and 6, the cyclins that interact with the kinase Cdk1 to activate

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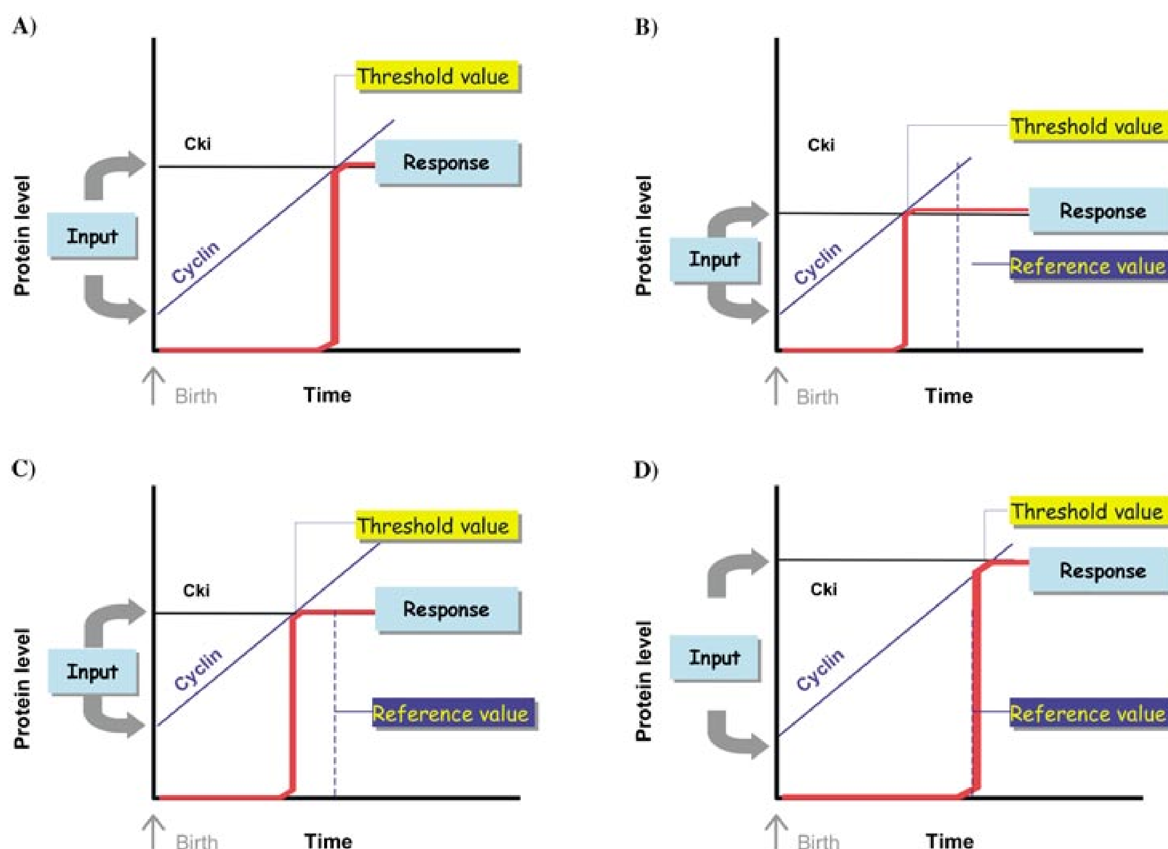

Figure 1. *A biochemical threshold is a major regulatory element within a blueprint which describes cell-cycle progression in budding yeast. A schematic representation of the threshold mechanism controlling the G1/S transition in budding yeast and how it can be modulated by changing the level of one or both threshold components is shown. See text for details.*

DNA replication. Threshold execution is made irreversible by degradation of Sic1, primed by a multiple phosphorylation dependent on Cln1, 2–Cdk1.[51] The newly described two-threshold control of cell-cycle transition from G1 to S is able to reconcile a wealth of literature data and its modeling and simulation analysis correctly predict major features of the G1 to S dynamics.[52]

We have also collected evidence which suggests that both thresholds cooperate in glucose modulation of Ps, a hallmark response of the cell cycle to changing growth conditions. It is in fact well established that yeast cells growing in ethanol-supplemented media have a critical Ps smaller than cells growing in glucose-supplemented media.[39,41] Glucose-induced increase of Ps is severely hampered, but not destroyed, when cells are unable to monitor extracellular glucose while being able to utilize it. However, when genes encoding components of both thresholds are deleted, carbon source modulation of Ps is completely lost. This finding suggests that Ps is set by both intracellular and extracellular glucose. This implies that the G1/S module is controlled by being wired to both a signal transduction module that senses extracellular glucose and a second module, most likely glucose metabolism itself, that is dependent on intracellular glucose. While dysfunction of a regulatory module only partially affects glucose-modulation of Ps, disruption of components of both thresholds, that is, the actuator module, completely abolishes glucose control over Ps.

Molecular Alterations of Proteins Involved in Cell-Cycle Regulation in Transformed Mammalian Cells: Focus on the Module Controlling G1 to S Transition

The experiments reported above have stressed the relevance of two sequential Cki dependent thresholds, the first of which is modulated by growth, as the key event for entry into S-phase. We were prompted therefore to investigate how transformation affects putative molecular determinants of the two thresholds which control entry into the S-phase. The first one involves Cdk4 and cyclin D and the second Cdk2 and cyclin E.[27–29]

A large body of literature on the alteration of expression, localization, function, and interaction ability of Cdks, cyclins, and Ckis in transformed cells has appeared in recent years. Without any attempt to be exhaustive, these alterations include:

- Amplification, over-expression of Cdk4 (and Cdk6);[53,54] mutations in *cdk4*- and *cdk6*-encoding genes that reduce the ability of the proteins to be inhibited by Ckis.[53–55] These mutations lead to misregulation of Cdk4 and 6 kinase activ-

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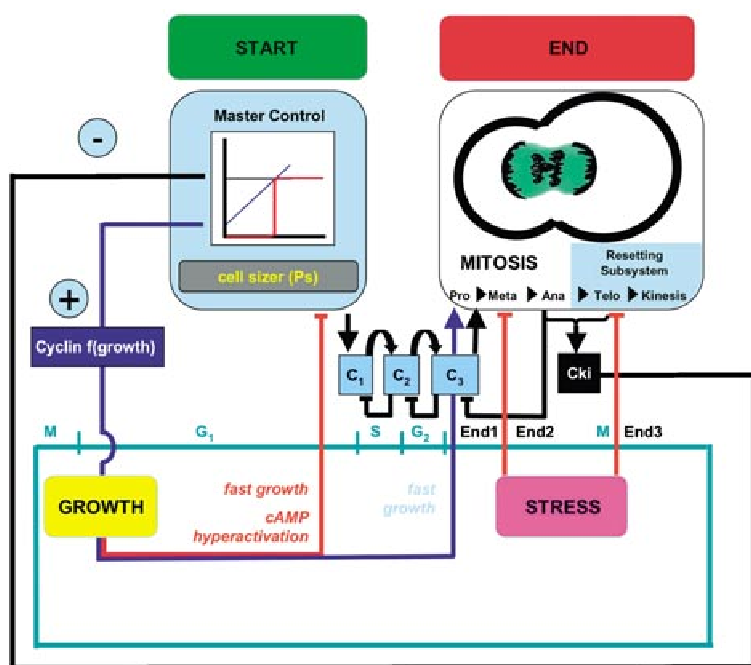

Figure 2. Modeling the sequential interconnections between the main modules of cell-cycle progression. The cell-cycle blueprint is given by three functional units: a Start function that allows the onset of the S-phase when a critical protein level has been achieved, that is, when the Far1–Cln3 threshold is achieved, followed by a cascade of three cyclin subsystems (indicated as C1, C2, C3) and an End function that comprises the events from the onset of mitosis to cell division. ⊕ and ⊖ indicate that cyclin and Cki act as the positive and negative component of the threshold mechanism, respectively.

ity, dramatically affecting the progression in the cell cycle and the onset of malignant transformation, as confirmed by studies with mouse models.[56,57] Genetic or epigenetic alterations of Cdk2 and Cdk1 have rarely been described in human tumors.

- Over-expression,[58] gene amplification, polymorphism of cyclin D1 and, with lesser frequency, cyclin D2 and D3.[59,60] Interestingly also a polymorphic germ-line mutation of the cyclin D1 gene, which is possibly involved in human carcinogenesis, has been reported. It gives rise to a cyclin D1 protein with a longer half-life, which possibly allows an over accumulation of cyclin D1 in the cell, and in turn promotes increased cell proliferation, and thereby elevated risk of cancer development.[61,62] Amplification, over-expression or altered post-transcriptional regulation of cyclin E1 and 2, cyclin A1 and 2 and cyclin B1 and 2 have also been reported. Notably over-expression of cyclin A2 is associated with a poor prognosis in several types of cancer and cyclin A1 is highly expressed in acute myeloid leukemia and testicular cancer.[63]
- p21Cip1 has not been found to be mutated in human cancers: it is a direct target of p53, its function in tumors is strictly correlated with p53 status, the most commonly mutated gene in human cancers.[64] The homozygous loss or silencing of the p27Kip1-encoding locus is extremely rare, but decreased p27Kip1 protein expression and/or enhanced degradation has been reported for a number of human tumors and has been correlated with poor prognosis and tumor aggressiveness.[65] Besides, altered p27Kip1 localization, from nucleus to cytoplasm, it has been reported in carcinomas[66–68] and has been shown to correlate with a more aggressive clinical behavior and decreased survival. Cytoplasmic localization may result from phosphorylation of p27Kip1 by the oncogenically activated kinase Akt/PKB[66–68] or by sequestration of p27 in the cytoplasm by cyclin D–Cdk4/6 complexes. Both Ras/Raf/Mek and PI3K/Akt pathways can thus regulate p27Kip1 localization by their direct effect on cyclin D levels. Point mutations, and deletions of genes encoding Ink proteins as well increased Ink protein degradation have also been reported.[60,69–72]

The relevance of Cdks, cyclins and Ckis for transformation has been further supported by studies on transgenic animal models.

- Targeted disruption of Cdk4 in mice delays cell-cycle entry and has been associated with increased binding of p27 to cyclin E–Cdk2 and diminished activation of Cdk2, accompanied by impaired Rb (retinoblastoma) phosphorylation.[73] The same *cdk4*$^{-/-}$ mice were also significantly smaller indicating that normal Cdk4 expression is critical for optimal growth of the organism.[74] On the contrary over-expression of Cdk4 in mice results in increased proliferation, hyperplasia, and hypertrophy.[56,57,75] As seen in mice, coexpression of cyclin D and Cdk4 coordinately increases rates of both cell growth and cell-cycle progression, resulting in faster rates of cell division.[76] In this scenario, deletion, inactivation and delocalization of p27, resulting in a loss of its activity, induces a major activation of cyclin D–Cdk4 complex and increased growth and proliferation.
- Transgenic mouse models in which the cyclins D1, E1 and A1 were over-expressed, show increased proliferation and tumor susceptibility.[77–79] Cyclin D1-deficient mice are smaller, as seen in *cdk4*$^{-/-}$ mice, which also indicates proliferation and/or growth defects.
- The involvement of the four members of the Ink4 family in the regulation of the cell cycle in normal and cancer cells is being elucidated by genetically modified mice.[80] This gives us a more clear representation of the role of these cell-cycle regulators in normal and pathological processes. Mice deficient for both p16Ink4a and p19ARF are viable but highly prone to tumors, and succumb to lymphomas and fibrosarcomas early in life.[81] This indicates that Ink4a encodes growth inhibitory proteins that act upstream of the Rb and p53 proteins. Genetically modified mice for the Kip/Cip family have clarified the role of this family in the tumor suppressor pathway, and in identifying p27 as a haploinsufficient tumor suppressor. Tumor suppression capacity of p27 is critically dependent on the absolute level of p27 expression suggesting that p27 acts as a rheostat rather than

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as an on/off switch to control growth and neoplasia.[82] Another important feature of p27 null mice is the appearance of a gigantism phenotype, indicating involvement of p27 in tissue growth and proliferation.

When considered in the frame of the two thresholds model of the cell cycle presented above, the great majority of the molecular alterations discussed appear to converge to change the setting of two thresholds controlling entry into the S-phase and to facilitate overcoming it. Coordination between growth and cell-cycle progression should then be altered with a tendency to yield smaller cells. In fact either a reduction in the level of the Cki (Figure 1 B) or an increase in the level of the cyclin activator subunit (Figure 1 C) should result in the anticipated execution of the threshold. This could event put a significant selective constrain on the clonal evolution of mammalian cells.[41,47]

Signal Transduction Pathways Involved in Cell-Size Control in Multicellular Organisms

The findings summarized above and the hypothesis that transformation may alter coordination events that ensure cell size homeostasis, make the brief summary of the general picture emerging from the available data on the regulation of cell-cycle control in cells of multicellular organisms relevant. As discussed in a previous paragraph, evidence collected in organisms ranging from fission yeast to unicellular algae, from flies to mammalian cells, indicate that entrance into S-phase (or eventually into M-phase) requires the attainment of a critical cell size.[39–42] Yeast and mammalian cells may share both nutrient and external signaling control over cell-cycle entry.[40] The relevance of these two classes of regulatory controls might be widely different in different species and cell types, up to a point, when one or the other mechanism may become cryptic under laboratory conditions. Our recent findings that in yeast, glucose affects the setting of the cell sizer mechanism through a G protein-coupled receptor system, independently of its energy-supplying role,[45] point in this direction. An increasing number of reports have shown a similar way to regulate cell growth and cell division in *Drosophila*. In fact genetic studies have highlighted the role of the insulin pathway in regulating cell and tissue growth. Loss-of-function mutations as positive regulators of this insulin pathway result in a reduction in the cell size and slower cell-cycle progression. On the contrary, over-expression of these genes, or loss of function of inhibitors of this pathway (PTEN) induce increased cell size without altering the duration of the cell cycle (under optimal condition). These results have been explained by a shortening of the G1-phase in parallel with an increase in the length of the S and G2-phases. Detailed analysis of the insulin pathway indicates that it is involved in two fundamental processes: regulation of the translational capacity of the cell and therefore the rate of mass accumulation on one side, and induction of cell-cycle progression and therefore cell division on the other. Recent results indicate that Akt, a downstream target of the insulin pathway, has the ability to positively regulate the level of Cdk4 and cyclin D1, and negatively regulate that of p21 and p27 inhibitors.[83] Studies of these proteins in different model organisms, such as *Drosophila* and mice, have underlined the capacity of these cell-cycle regulators to coordinate cell growth and proliferation. These and other observations have led to the idea that cyclin D and Cdk4 can act both as a growth sensor and as a growth driver (reviewed in ref. [84]).

Ras-Transformed Cells Show Distinctive Alterations in the Molecular Circuits Controlling Entry into the S-Phase

The availability of an experimental system in which to investigate specific transformation events affecting the control of entry into S-phase could enable the pursuit of this line of thought. Mutation of the *ras* gene is a critical event in the onset of different malignant phenotypes. Ras proteins are intracellular switches the activation states of which (i.e., their binding to GDP and GTP) controls downstream pathways leading to cell growth and differentiation. Their activation state depends on the competing action of GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF). Altering this fine balance by deregulation of either GAP or GEF activity might result in hypo- or hyper-activation of downstream pathway(s), so that, for instance, over-expression of a GEF or inactivation of a GAP coulld both result in cell transformation.[85,86]

Research performed in our laboratory indicates that a single amino acid change within the catalytic domain of the mammalian GEF, $CDC25^{Mm}$, turns the molecule into a dominant negative protein (GEF-DN).[87] The mutant is able to efficiently displace wild-type GEF from $p21^{ras}$ and to give rise to a stable Ras–GEF binary complex, due to the reduced affinity of the nucleotide-free Ras–GEF complex for the incoming nucleotide.[84] This "Ras-sequestering property" can be utilized to attenuate Ras signal transduction pathways in mouse fibroblasts transformed with oncogenic *ras*. In fact over- expression of the GEF-DN in k-*ras* transformed fibroblasts, derived by transfection of an activated k-*ras* gene into NIH/3T3 cells, strongly reduces intracellular Ras–GTP to a level similar to that observed in untransformed fibroblasts. Accordingly, stable cell lines of k-*ras* transformed fibroblasts expressing GEF-DN revert to wild-type phenotype with respect to morphology, anchorage-independent growth and stark reduction of tumor formation in nude mice.[88]

The above described cell lines represent a useful model system in which to study the Ras-dependent alteration of regulatory circuits that lead to cellular transformation in mouse fibroblasts. Studies are presently ongoing in our laboratory by using a combination of transcriptional profiling, molecular physiology and nutritional perturbations to reconstruct the regulatory network controlling the G1 to S transition in normal (NIH/3T3), transformed (NIH-*ras*), and reverted cell lines (NIH-*ras*-DN). NIH-*ras* cells show enhanced proliferation possibly because of their sustained ability to divide at higher cell densities, conditions in which normal NIH/3T3 cells stop growing. Expression of such an increased growth potential requires a high initial glucose concentration (25 mM) in the medium,

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since the selective advantage of NIH-*ras* cells was lost upon growth in suboptimal glucose concentrations (1 mM), as well as a sustained Ras pathway activation since GEF-DN expression dramatically reduces proliferation ability. Notably, the transcriptional profile of NIH-*ras*-DN was similar, though not identical to that of the parental cell line, indicating that NIH-*ras*-DN cells are not simple molecular phenocopies of the parent cells, despite sharing many similar phenotypic properties. NIH-*ras* cells show alterations in the G1/S transition and control of cell size compared to normal cells. In NIH-*ras* cells the up-regulation of several components of the thresholds governing the G1/S transition combined with the activation of the Akt pathway may indeed result in larger cells, as observed[89](Figure 1D). Given the dual role played by Cki in regulating Cdk activity (promotes complex formation in the cytoplasm and inhibits kinase activity in the nucleus), nonlinear and nonintuitive dynamics are generated among the components of the threshold. Measurements of the level of cyclins, Cdk and Cki, of the localization, formation and activity of each Cdk complex, as well as simulation of the dynamics of the network are currently under way and will be used to clarify the differential regulation of this important network in normal vs. transformed cells.

Toward a New Strategy for the Discovery of Anticancer Drugs

The understanding of regulatory circuits that control complex phenomena, such as cell growth and division, differentiation, signal transduction pathways and their flexible yet robust coping with ever changing environmental stimuli, appears relevant in developing a new generation of selective and effective drugs, that are much needed to combat major multifactorial diseases such as cancer. A chief merit of the systems biology approach in the study and control of physiopathological systems lies in shifting attention from individual molecules to networks of molecules. Such networks, and the properties inherent in the networks, but not present in their individual components, may thus be putative targets for drug discovery and therapeutic intervention. The systems biology approach will then be effective at different levels. It can:

- Contribute to target identification by highlighting the topology of regulatory networks and suggesting "fragile" components to be used as targets.[19] For instance making use of detailed models of signal transduction pathways (see for instance ref. [90]) to identify possible novel attack points that can be used as drug targets. The concept of modularization is here very important. Appropriate wiring of a signal transduction pathway within a larger regulatory network, that is, when the pathway becomes a module within a larger system, could allow the failure of a therapeutic approach that might otherwise be nonintuitive when analyzed out of the appropriate cellular or tissue context to be rationalized.
- Make use of biological modeling to integrate diverse sets of data to support the drug discovery process through the exploration of hypotheses, in silico. For instance, hypothesis-driven approach to drug discovery could address selection of preclinical programs by exploring parameter space of experimental variables, allow direct comparisons, and predict outcomes while at the same time reduce expensive "wet-lab" experiments.[91]
- Make use of in vivo high throughput (systems level) metabolic profiling to model the metabolic response of different, specialized cells to changes in the environment, especially with respect to the interaction between metabolism of endogenous and exogenous compounds.[92]
- Contribute to the development of personalized therapy by rationalizing the optimal choice of drugs as well as optimal dosage and scheduling by using knowledge of cellular and intercellular dynamics, based on the identification of specific targets for each individual patient.[19]

Figure 3 shows selected signal transduction, regulatory cascades and metabolic modules involved in tumor formation and a low resolution, partial wiring among them. The cell-cycle module is represented within the nucleus. The two thresholds governing the G1/S transition as well as Cdks and cyclins acting as their inputs are shown. Direct (positive and negative) regulatory links from growth factor and signaling pathways to Cdk and cyclin accumulation are illustrated. One of the major pathways mediating growth factor action, the Ras pathway, is indicated. For clarity, explicit Cki and cyclin input is shown only for the first threshold. Metabolism is directly related to nutrient supply as well as being regulated by growth factors and hormones. Energy metabolism in turn affects, through still largely unknown mechanisms, cell-cycle execution. A possible direct link from nutrient and energy supply to the cell cycle as well as a putative feedback of cell cycle on energy metabolism is shown by dotted lines. Energy metabolism provides ATP to cells, while mitochondrial malfunctioning may increase apoptosis and hence decrease replicative lifespan. Uncontrolled cell proliferation results from the inability of a cell to respond to negative growth signals and/or to become independent of positive growth signals. Tumor growth results from the combined uncontrolled proliferation potential and escape from apoptosis, increased angiogenesis and cell motility (see refs. [13, 14, 17] for more details).

The acquired ability of *ras*-transformed fibroblasts to proliferate even under conditions of growth factor shortage and/or high density[89] is a robust property of transformed cells that maintain proliferation ability regardless of perturbations such as growth factor withdrawal or high cell density (red boxes). At the same time, this acquired robustness comes at the price of an acquired fragility, that is, the exquisite sensitivity of NIH-*ras* fibroblasts to shortage in nutrient and energy supply (i.e., growth in low initial glucose concentration, white box). Normal NIH/3T3 cells cope with nutrient shortage most likely by gaining a larger fraction of their energy budget by oxidative means. NIH-*ras* cells, on the contrary, loose sustained ability to enter S-phase and die by apoptosis.

Such an acquired fragility could make tumor cells more vulnerable to an appropriate dosage of a drug limiting glucose uptake or utilization, such as 2-deoxy-D-glucose (2-DG) and 3-

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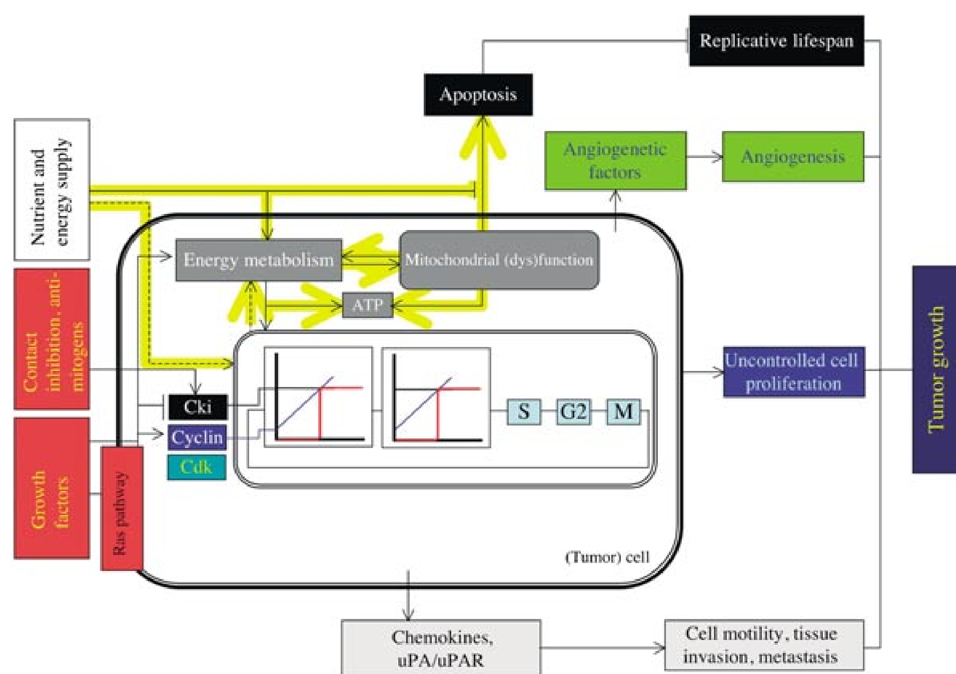

Figure 3. Low-resolution, partial blueprint of the cell-cycle module—represented within the cell nucleus—with different modules affecting tumor growth. Uncontrolled cell proliferation results from the inability of a cell to respond to negative growth signal and/or to become independent of positive growth signals. Tumor growth results from the combined uncontrolled proliferation potential and escape from apoptosis, increased angiogenesis and cell motility.

bromo-pyruvic acid, than normal cells. Since NIH-*ras* cells dye by apoptosis, the apoptosis module could be a valuable target for a combined therapy, as indeed suggested by recent reports on cancer cells grown in vitro.[93–96] On the contrary, no attempts should be made to combine a drug which limits glucose uptake or utilization, with inhibition of the Ras pathway. In fact down-regulation of the Ras pathway eliminates tumor-induced fragility towards low glucose regimens. This effectively eliminates the network that is the target of the first drug, which would thus become ineffective.

The rationale behind the pharmacological use of 2-DG in pharmacological approaches have been largely based on the Warburg hypothesis[97] that cancer cells are inherently glycolytic. While this is still the object of debate (see ref. [98] for a recent review), it should be stressed that what has to be targeted is not glycolysis per se, rather the transformation-dependent fragility of the interconnections among the metabolic, cell cycle, and apoptosis modules (highlighted in yellow in Figure 3). Such fragility appears to be quite diffuse among cancer cells. Several recent reports have in fact shown that glucose deprivation of human cancer cells cause cytotoxicity, as well as activation of multiple signal transduction and gene expression pathways involved in maintenance of phenotypic characteristics associated with malignancy, including angiogenesis and expression of cellular homologues of oncogenes.[93,99–101]

Consistently, combined treatment of tumors in vivo, mouse xenograft models and human trials, have indicated that inhibitors of glycolysis could have widespread application within combined therapies for a variety of tumor types. 2-DG was shown to be able to enhance the effect of radiation therapy.[102] Other examples of combined therapy which use 2-DG in vivo were reported with chemotherapeutic agents such as paclitaxel and adryamicin, which work as antiangiogenic and or antiHIFα agents. This combination was found to increase the hypoxic state of these tumors, which lead to enhanced efficacy of 2-DG and reduced tumor mass, with direct influence on proliferation of tumor cells.[103,104] Another important application related to a modified form of 2-DG is the diagnosis, staging and therapy monitoring of tumors by using fluorine-18 fluorodeoxyglucose combined with positron emission tomography (PET) imaging. This technique provides a noninvasive way to asses tumor metabolism and gives significant information on the effects of combined cancer therapy.[105–107]

It is well known that the development of a malignant tumor requires "cooperation" by other, nontumor tissues, for instance the formation of new blood vessels that allow appropriate nutrient and oxygen supply to the developing tumor (Figure 3). When the same hierarchical, modular approach described above at the single cell level is extended from the transformed cell to the tumor as a whole, appropriate wiring of input and output from different cells and tissues and the study of the resulting dynamics, becomes of even greater importance and holds promise for the development of effective drug discovery approaches and more efficient therapeutic protocols.[19]

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