

Problems & Paradigms

Cell size control - a mechanism for maintaining fitness and function

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Abstract

5 The maintenance of cell size homeostasis has been studied for years in different
cellular systems. With the focus on ‘what regulates cell size’, the question ‘why cell
size needs to be maintained’ has been largely overlooked. Recent evidence indicates
that animal cells exhibit nonlinear cell size dependent growth rates and mitochondrial
10 metabolism, which are maximal in intermediate sized cells within each cell
population. Increases in intracellular distances and changes in the relative cell surface
area impose biophysical limitations on cells, which can explain why growth and
metabolic rates are maximal in a specific cell size range. Consistently, aberrant
increases in cell size, for example through polyploidy, are typically disadvantageous
15 to cellular metabolism, fitness, and functionality. Accordingly, cellular hypertrophy
can potentially predispose to or worsen metabolic diseases. We propose that cell size
control may have emerged as a guardian of cellular fitness and metabolic activity.

Introduction: Cell size control models

Cell size is closely associated with cell division, as most cells need to grow and increase their size before they can divide. Research on cell size has mainly focused on identification of molecular mechanisms linking growth and cell cycle, particularly if and how key cell cycle phases, such as initiation of DNA synthesis, are dependent on cell size. This body of work has been extensively reviewed (see [1-3]). Another area of interest has been the development of phenomenological models how cell size homeostasis is achieved. The most prominent models, at least until recently, were the “sizer” and “timer” models. The “sizer” model assumes that the cell begins to divide once it reaches a critical size, whereas the “timer” model suggests that the cell grows for a specific amount of time prior to dividing [4]. These models require that cells can either measure their size or time, respectively. As neither of the models has gained universal acceptance, recent interest in bacterial size control has resulted in resurrection of the ~40 years old “incremental” [4] a.k.a. “adder” model [5]. The “adder” model postulates that cells add a constant volume (or mass) between birth and cell division. The constant volume addition means that smaller cells add more volume, in relative terms, during each cell cycle compared to larger cells. Thus, size homeostasis is automatically achieved by mean reversion as the daughter cell size corrects itself towards the normal size. In bacteria as well as budding yeast, the observed cell growth is consistent with the adder model [5-7]. There is less data from other model organisms but at least plant cell growth does not fit perfectly with the adder model [8].

The current models have been very informative, but they depend on cell proliferation. Instead, most animal cell types in vivo are terminally differentiated and not proliferating, yet they maintain size homeostasis. Non-dividing cells clearly cannot use cell division cycle for size adjustment, instead they likely rely on the balance between biosynthesis and degradation [9]. Such regulation may be even counterintuitive as cell size can increase when protein biosynthesis is reduced, provided that there is an even more pronounced reduction in catabolism [10]. There is a common misconception that non-proliferating cells are metabolically inactive. While this may be true for quiescent lymphocytes [11,12], some fibroblasts display metabolic activity comparable to proliferating cells [13], and neurons are well-known for their high metabolic activity, which is partly required for cellular “housekeeping” [14]. Hepatocytes are yet another example of a metabolically active but non-proliferating cell type.

A key unresolved question is whether cell size control is active or a passive consequence of underlying growth-laws that dictate the rate of cell division or initiation of DNA replication in proliferating cells [15]. The adder model favors a passive mechanism for the maintenance of cell size and size distribution within the cell population. On the other hand, the apparent uniformity of cell sizes (including non-dividing cells in most animal tissues), suggests that cell size homeostasis is important and should thus be actively maintained [16]. The main limitation of the adder model is that it cannot easily explain why larger cells would add less (in relative terms) and smaller cells add more volume (again in relative terms) during the cell cycle [17]. To achieve constant volume addition, abnormally small and large cells would require a cell size or growth rate sensing mechanism that is somehow linked to cell cycle machinery to maintain size uniformity [16]. Slowing down cell cycle transitions for smaller cells would result in an adder-like behavior as small cells would have time to grow for a longer time before initiating DNA synthesis or mitosis. In addition to the

mechanisms controlling cell growth and/or division rate, cell size homeostasis could potentially also be achieved through size dependent cell death [18].

5 The existence of a mechanism that couples animal cell size to cell cycle and involves active and cell-autonomous size sensing has been often challenged. In some models, where growth factor and mitogen levels are fully controlled, growth and cell cycle can be uncoupled [19]. While these models allow cell size to be studied independently of proliferation, it is possible that the manipulation of the extracellular factors has downstream signaling effects that turn on or off the mechanism(s) that normally couple(s) growth and division (Table 1). Based on available evidence, at least animal cells have an apparent ability to sense their size [16]. It remains to be established whether this apparent size sensing is universal among organisms and if there is a unifying molecular mechanism, such as volume dependent dilution of a cell division regulatory protein [20] or growth rate [21]. However, it cannot be excluded that cell size is not monitored by any means and is simply an emergent property resulting from growth and cell division homeostasis [9,22].

Cell size connects to cellular growth rate

20 The relationship between size and growth rate has been the prime inspiration for biologists working on size control. Although the correlation between growth rate and cell size was observed in bacteria already in the 1950's and 1960's [23-25], there is still no clear agreement how growth and size scale with each other. This lack of consensus can possibly be explained through fundamental differences between prokaryotes, single-celled eukaryotes and multicellular organisms, but also by different means of measurements (e.g. mass, volume or an indirect proxy such as light scattering or an amine reactive fluorescent probe), data fitting (e.g. single or multiple fit across the cell cycle) and normalisation (e.g. absolute or relative growth). For example, impressions drawn from absolute and cell size-normalised data regarding growth rate can be very different although the underlying data is identical (see Fig. 1A & B). Therefore, the lack of standardized methodology may have hampered the progress in this field (Table 1).

30 While a faster growth rate typically results in larger cells, this is true mostly if populations of cells growing under different nutrient conditions are compared. Instead, if one compares cells within a population, a different picture typically emerges. For example, in budding yeast the correlation between cell size and growth rate is negative, bigger cells growing slower than smaller ones despite having more ribosomes for translation [26]. Consistently, yeast growth is fastest early in the cell cycle and growth substantially slows down at the G1/S boundary [27]. In multicellular organisms, recent evidence has indicated that growth is nonlinear and declines in largest cells [8,28-31]. Animal cells seem to initially increase their growth rate with increasing cell size (indicative of exponential growth) after which growth plateaus or is even reduced (Fig. 1A & B).

Metabolism, not just ribosomes, drives growth

45 The early observations of exponential bacterial growth were explained through ribosomal content and, consequently, translation being increased in larger cells [25]. It has been reported that ribosome content correlates with the growth rate in animals as well [32], and that cell growth is limited by protein synthesis [33]. In other words, translation has been seen as the main factor determining cell size, which was likely also part of the reason leading to the dominance of a TOR pathway-centred view on cell size control [34,35]. However, there are potential issues with this ribosome-

oriented thinking for explaining cell size control. First, while the number of ribosomes typically increases with cell size and this supports faster growth, it does not necessarily mean that ribosomal content is the key limiting factor for growth. As mentioned above, growth is commonly nonlinear and declines in largest cells [8,28-31]. Instead, one should ask, ‘what limits ribosomal activity in larger cells?’ Second, while ribosome amount or translational activity can limit cell size increase, it does not readily explain why cells prefer to be of certain size.

Rates of transcription and translation depend on the metabolic activity of the cells [36,37]. In slow-growing yeast cells ribosome content appears to be limiting growth whereas under other conditions, oxygen dependent conversion of nutrients to ATP, appear more important [26]. Consistently, the amount of ATP generated equals approximately the energy requirements for protein synthesis across tens of different cell lines [30], highlighting the homeostasis in cellular energy production and consumption for biosynthesis, regardless of absolute ribosome number. On an organismal level, the metabolic rate, which is commonly measured as total oxygen consumption, is one of the best-established factors defining growth rate. In 1934 Ludwig von Bertalanffy suggested that an animals’ growth rate was proportional to its metabolic rate. The metabolic rate declines with increasing organismal size, which is known as metabolic allometry [38]. Since allometry should also apply to individual cells [39], the cellular growth rate is expected to decline with increasing cell size. As mentioned earlier, evidence particularly from mammalian and plant cells support this concept that growth rate stagnates or is even reduced in larger cells [8,28-31]. These observations are consistent with the idea that while ribosomes can limit growth, the true limitation to cellular growth rate and cell size is the metabolic rate required to support translation and other growth processes.

Cell size and cellular fitness & functionality

Advances in cell size control models and mechanisms have been tremendous. Yet, we may have been sidetracked from the most important question: Why do cells need to maintain their size? While cell size is often mentioned to be important for cellular functionality from development to aging [40,41], it has not been widely appreciated how cell size specifically affects the organismal fitness and function, especially in multicellular organisms.

A key finding from the bacterial in vitro evolution experiments performed in 1990’s was the rapid co-evolution of cell volume and cellular fitness (as measured by growth rate) [42]. This link with cell size was observed possibly because the cells were directly selected to maximise their growth rate [15]. However, it is noteworthy that while there is a linear relationship between size and fitness/growth rate over a large range of bacterial cell sizes [42,43], specific environments or very large increases in cell size do reduce the fitness benefit of increased cell size in bacteria [43].

Fitness in animal cells is commonly measured as proliferative capacity using either non-quiescent cells in culture [44] or during tissue development such as in cell competition [45]. However, it is important to note that proliferative capacity (evolutionary fitness) may not be that relevant for individual cells in multicellular organisms as most cells within a tissue do not proliferate, and co-operate rather than compete. In this review we use fitness to describe the ability of cells and organisms to perform their specific function and their adaptability to cope when challenged (akin of physical fitness in athletes) rather than the more narrow evolutionary interpretation (reproductive fitness).

Because cellular fitness and the growth rate are connected in most in vitro experiments using proliferating cells, the cell size scaling of growth rate is likely to reflect the cell size scaling of cellular fitness. It is important to stress that ribosomal [26] or mitochondrial [46] functionality, rather than the quantity, appear to define the maximal growth rate and optimal fitness. While the scaling of organelles and intracellular structures with cell size has been appreciated [47], the activity of organelles does not always scale with size [46,48]. Therefore, simply having more ribosomes or mitochondria, or a larger spindle, may not always be beneficial for the cell [26,46,49] and may come at a cost.

We recently demonstrated that in animal cells the intermediate cell size within a population (where growth rate is the highest) is linked to maximal fitness and optimal mitochondrial function [46]. This readily suggests that cell size is directly coupled to metabolism, i.e. metabolism is cell size dependent. At the same time, changes in metabolism are likely to be required to maintain and adjust cell size resulting in feedback between size and metabolism. Indeed, such a feedback between cell's physical dimensions and metabolic capacity is considered a requirement for active cell size control [16].

Connecting metabolism and fitness to cell size in non-dividing cells

The relationship between cell size and metabolic activity is likely to have consequences also in non-dividing cells. For example, as increased cell size is associated with reduced oxidative metabolic capacity, organisms are likely to be organized so that the cells which most dependent on high oxidative metabolism are small. As so often in biology, there are rules and exceptions. In skeletal muscle cells, there is an inverse relationship between oxidative metabolism and muscle fiber size. This is highlighted in muscles' limited capacity for simultaneous increase in strength through hypertrophy and endurance capacity through increase in oxidative metabolism [50,51]. Consequently, the perceived fitness of the muscle cells depends on whether one measures fitness as an ability to perform resistance or endurance exercise. There are also conditions where mitochondrial activity declines when cell size is reduced. Muscle sarcopenia and neuronal atrophy during aging are probably the best examples. While the inverse relationship between size and metabolic activity is broken in these cases, cellular fitness correlates with the normal (optimal) cell size. Small but highly oxidative muscle fibers also illustrate the fallacy of thinking only in terms of growth rate. Small fibers are highly active biosynthetically but they also display a high turnover rate [50]. However, a major challenge is that we do not properly understand the causalities behind these relationships between cell size and metabolic activity.

Challenges in validating the cell size and fitness hypothesis

Different cell types display a characteristic cell size and intuitively this appears to be related to their function (Fig. 2). For example, leukocytes need to be small to be able to travel through tiny capillaries, whereas neurons are capable of faster signal propagation when relying on one long cell instead of several short ones. However, it may not be possible to define one single key function for each cell type, as cells are often pleiotropic. Thus, analysis of how fitness and functionality scales with cell size in certain cell types may be challenging.

Experimental validation of cell size dependent fitness and functionality in animal cells will be hampered by difficulties in technical and experimental design (Table 1). Ideally, one should obtain high-resolution single-cell measurements of cellular functions and cell size under unperturbed conditions (as done in Fig. 1A & B)

and then use the natural variability in cell sizes to observe how functions scale with cell size. However, using this approach cell cycle dependent changes may mask the cell size effects and a substantial number of single-cells need to be analysed to separate cell size and cell cycle effects [46]. While this may be done in cultured cells, analysis of tissues is likely to be challenging. As an alternative approach, one should engineer mutants spanning a wide range of cell sizes without compromising normal cell cycle progression. All this makes it challenging to obtain non-circumstantial evidence that is needed to unravel the true relationship between cell size and cellular growth, fitness and functionality. We also need to acknowledge that there is unlikely to be one universal relationship that is applicable to all cell types under all circumstances. Currently, some of the best evidence for size dependent fitness and functionality comes from polyploid cells, which display vast increases in cell size.

Polyploidy in cells and animals reduces fitness but may improve adaptation

Ploidy is defined as the number of sets of chromosomes in a cell and the resulting DNA content is typically directly proportional to cell size. Thus, polyploid cells can potentially provide evidence for the importance of cell size, especially if cell size and ploidy effects can be distinguished. While ploidy and cell size correlate well, the cellular environment nevertheless plays an important role in determination of the final cell size even in polyploid cells [52,53]. A large body of experiments in yeast indicate that changes in ploidy and, consequently, cell size are typically detrimental [54], but under changing conditions increased ploidy may improve adaptation [55]. This may be related to the assumption that cell size needs to be adjusted for each condition to provide maximal fitness and to optimize organismal survival. However, as a consequence of polyploidization, dosage sensitive genes rather than physical size could explain cellular fitness effects [56,57]. We also need to keep in mind that yeast is a unicellular organism and not all conclusions may be applicable to mammalian and other multicellular organisms.

The difference in animal sizes is mainly due to the difference in cell number rather than cell size. This suggests that maintaining a specific cell size may be more beneficial (or at least easier) than to generate large organisms by increasing cell size. Nevertheless, it is possible to select or engineer animals with abnormally large cells by taking advantage of a ploidy increase. These animals can be fully polyploid or may contain polyploid cells in otherwise normal diploid tissue. Such animals could be very useful for analyzing how cell size affects organismal functionality, for example, how do fewer large cells compare to many small cells in the metabolism or physiological function of a tissue. So far this has not been widely done. In the 1940's Gerhard Fankhauser generated polyploid Eastern newts [*Notophthalmus (Triturus) viridescens*] by heat modulation of the fertilized eggs. His work established the correlation between ploidy and cell size and also revealed that organ and organismal size control is distinct from cell size control. Curiously, he observed that the triploid newts with 50% larger cells performed poorly in learning studies, indicating cognitive problems associated with larger cell size [58]. Further work has attempted to generalize these findings by correlating cell size with brain complexity in other amphibian species and thus providing supporting evidence that too large cell size is a burden for brain function [59]. More recently, comparisons of diploid and triploid animals has revealed that especially in aquatic environments triploid animals tend to have a reduced metabolic rate, despite maintaining same organismal size [60], suggesting a potential fitness disadvantage for abnormally large cell size.

Polyploidy can also be found in a subset of mammalian tissues even under

normal conditions. Whole organismal polyploidy in mammals is usually lethal, but polyploidization is common particularly in hepatocytes during development [61]. The increased DNA content and associated larger hepatocyte size is considered normal for liver for functional reasons that remain incompletely understood. However, there is a cost to polyploidization as polyploid cells may be genetically unstable [62]. Several stress factors may induce polyploidization in differentiated diploid hepatocytes, such as regeneration after partial hepatectomy, oxidative stress, metabolic imbalance, chemical damage, or viral infection [63] (Fig. 3A). Therefore, as with yeast, hepatocyte polyploidy can be used to increase genetic variability, which would help in adaptation to chronic stress and damage [64].

Many different genetic mouse models have been used to generate polyploid hepatocytes and other cell types [64]. We have taken advantage of cyclin-dependent kinase 1 (Cdk1), which controls DNA re-replication in polyploid hepatocytes during liver regeneration after partial hepatectomy [65]. Through a conditional deletion of Cdk1 in hepatocytes, we generated a mouse model with increased genome content and cell size due to the DNA re-replication phenotype [66]. As Cdk1 is essential for cell division, the resection of up to 70% of the liver mass (partial hepatectomy) followed by liver regeneration can be used to further increase cell size [66,67] (Fig. 3B & 3C). The benefit of this genetic model for analyzing cell size is that the animals are isogenic and that any direct effects of Cdk1 on cell size can be largely excluded by comparing to the Cdk1 null and wild-type livers before partial hepatectomy [67]. Analysis of these mice indicated that the relative expression of most mitochondrial genes, whether encoded by the mitochondrial or the nuclear genome, was reduced in large hepatocytes. This finding is consistent with metabolic allometry.

Our data also indicated that increased hepatocyte size and nuclear DNA content in hepatocytes is strongly associated with reduced expression of genes involved in lipid synthesis and increased glycolysis [67]. This implicates that in hepatocytes with increase in cell size and polyploidy, mitochondrial activity is reduced and ATP production likely compensated by enhanced glycolytic activity, resulting in substantial metabolic remodeling. Interestingly, the relationship between hepatic DNA content and lipid metabolism is comparable to non-alcoholic fatty liver disease patients who display a significant increase in polyploid hepatocytes [68]. Moreover, it has been observed that the increase in polyploidy in hepatocytes during NAFLD progression is due to excess of lipids causing increased oxidative stress [68], which is indicative of mitochondrial malfunction [69].

Supporting our *in vivo* observations, we noticed that mitochondrial functionality declines with increasing cell size in a manner dependent on mitochondrial dynamics, i.e., how mitochondria fuse and divide. This was observed in many different cultured cell types during the normal cell cycle, providing further evidence for cell size dependent metabolism [46,48]. Misregulation of oxidative phosphorylation has been associated with liver damage and liver failure (reviewed in [70]). While we have not yet experimentally challenged our animals to directly analyze the fitness of their liver, it appears that metabolism, nuclear DNA content and cell size during liver regeneration could be connected to organ and organismal fitness.

Biophysical mechanisms connecting cell size to fitness and functionality

Through what mechanisms could size influence growth, metabolism and cellular functionality? From a biophysical perspective, there are three common explanations for why too large cell size is not optimal: the structural integrity of the cells, the increased intracellular distances, and the reduced surface-to-volume (SV)

ratio. These factors can lead to metabolic inefficiency (Fig. 2) and cell death. These biophysical factors alone do not explain how or why each cell type has its own characteristic size. Cell type specific features, such as structure of the cytoskeleton or mitochondrial network morphology [48], likely influence how each cell type is dependent on biophysical limitations and what the optimal size for that cell type is.

First, structural integrity of cells could affect cell size and fitness. A stronger cytoskeleton is required to support larger cells [71]. Consistently, we have observed increased transcription of cytoskeletal genes in response to cell size increase [67]. However, the structural integrity of the plasma membrane might become limiting for the largest cells as the thickness of the membrane cannot scale with increasing cell volume. The mechanical strength of cells has not been widely studied in the context of cell size, but abnormally large hepatocytes appear unstable upon isolation, which is consistent with the idea of structural integrity limiting maximal cell size (M.J.C and P.K. unpublished).

Second, size induced increases in intracellular distances could limit almost all cellular functions. In larger cells, the diffusion and active transport of molecules and cellular components will take longer (Fig. 4A & 4B). As nutrients are consumed most in the center of the cell, where nucleus, most ribosomes and mitochondria are typically localized, this should create an intracellular gradient of metabolites, as has been observed with oxygen [72]. Consequently, the rate of biochemical reactions is reduced, thus making larger cells less metabolically active. Diffusion rates in cytoplasm (Fig. 4C) have been extensively studied and increase exponentially with distance (see [73] for review). Unfortunately, there is little quantitative evidence regarding the extent to which increased intracellular distances affect growth rate, metabolic rate or cell size, although mathematical models suggest that at least the maximal cell size is limited by intracellular transport [33,74]. Within the micrometer scale of most cells, this may not be intuitive, but considering a complete metabolic pathway, the diffusion distances required for metabolism could become significant. This is because the intracellular localization of some metabolic enzymes and organelles require metabolites to travel from one location to another before the whole metabolic route is completed (Fig. 4B).

Third, with increasing cell volume, the relative surface area declines (Fig. 4A & 4D). Reduced SV ratio is predicted to slow down nutrient and oxygen uptake and waste disposal on cellular and organismal level. Cells also need to maintain ionic gradients across the plasma membrane by actively pumping ions across the membrane to counteract ion leakage. For example, cells maintain high potassium and low sodium concentration inside the cell. As ion leakage through the plasma membrane depends on membrane area, cells with high SV ratio to use relatively more energy for upholding the ion balance.

Can surface-to-volume ratio control metabolism at the cellular level?

Our gene expression data from different sized hepatocytes did not show any systematic upregulation of ion transporters or other plasma membrane proteins in larger cells [67]. This suggests that abnormally large cell did not try to counteract the reduced SV ratio and nutrient uptake at the level of membrane proteins. Instead, the data indicated a strong downregulation of genes involved in lipid biosynthesis [67], consistent with the reduced plasma membrane area in larger cells. It is not known whether cells can sense their SV ratio, although the molecular mechanisms for this are easy to envision. For example, the levels of intracellular cholesterol, one of the lipids mainly located in the plasma membrane, could act as a signaling molecule reporting

the relative amount of plasma membrane in comparison to cytosolic volume. When excess intracellular cholesterol accumulates, it is sensed by the sterol regulatory element-binding proteins (SREBPs). SREBPs, in turn, control the activity of lipid synthesis pathways, including the mevalonate pathway, which produces cholesterol [75]. SREBPs could therefore couple SV ratio to membrane production [67].

There is still an ongoing debate if the SV ratio is behind the observed size-dependent metabolism at the organismal level (reviewed by [38]). Due to experimental limitations, there is mostly modeling based support for this on a cellular level. One potential way for studying the SV ratio would be comparison of polyploid cells obtained through inhibition of cell division to those obtained through cell fusion. Recent evidence from macrophages suggests that increasing cell size through cell fusion results in large amounts of excess plasma membrane [76]. In contrast, cells that become polyploid and large by normal growth in the absence of cell division should have less surplus membrane (although they may have excess lipids for building membranes) and thus substantially lower SV ratio compared to fused cells. In addition, mechanically restraining cells to specific shapes could be potentially used as an experimental system to study the influence of SV ratio on cellular metabolism.

The changes in SV ratio and intracellular distances also suggest that spherical cell shapes, where the relative plasma membrane area is minimized and intracellular distance from cell surface to the middle of the cell maximized, should be least optimal for a large cell. Consequently, complex cell shapes should be beneficial for large cells when active metabolism is required. The largest cell types in animals tend to be elongated (e.g., muscle cells) and often also branched (e.g., neurons). Yet, there are some individual cell types, like oocytes, which are very large and have spherical shape with a low SV ratio. How oocytes differ in their nutrient supply and metabolism is an interesting question in need of more research. Overall, the diversity of SV ratios seen in different human cells is extensive (Fig. 4D), and more systematic analyses of SV ratios and metabolic parameters in different cells may help elucidate the importance of SV ratio and intracellular distances. If cell size and growth is indeed limited by the SV ratio and/or the intracellular distances, these limitations may only become meaningful in larger cell types. This could partly explain the vastly different size-dependent growth patterns seen between small single-celled organisms and typically larger metazoan cells.

Emerging links between cell size and metabolic disease

As discussed above, we have potential biophysical mechanisms (the SV ratio) and an increasing understanding of molecular processes (mitochondrial metabolism) related to cell size changes. Yet, we should not forget how these are linked to the potential physiological consequences for being the wrong cell size. With age, many of us develop chronic diseases such as cancer, type II diabetes, cardiovascular problems, neurodegenerative diseases or obesity. Is cell size a driver, a passenger or both in metabolic conditions? While it is easy to claim that in most of these diseases there is a metabolic defect that drives the pathological phenotype, we should not ignore the evidence for cell size dependent functionality that has been slowly accumulating (reviewed in [16,48]).

Mitochondrial dysfunction in age-related metabolic diseases is well documented [77]. The mevalonate pathway may be yet another important cell size related player in some common metabolic diseases [75,78]. The mevalonate pathway affects cell size (Fig. 5A), changes mitochondrial functionality and mitochondrial dynamics (Fig. 5B) [46,79], and is required for normal autophagy [10], resulting in

cell size dependent changes in metabolism (Fig. 5C). Interestingly, cholesterol synthesis alone does not explain mevalonate pathway effects on cell size [10]. The mevalonate pathway is also responsible for making other products including heme, ubiquinone and steroid hormones as well as geranylgeranyl and farnesyl groups for post-translational modification of membrane proteins, which are used not only for plasma membrane, but for other processes as well, including mitochondrial electron transport. Nevertheless, the lipophilic nature of the mevalonate pathway products further suggests that the metabolic control of cell size is linked to membranes as suggested by work in prokaryotes [80,81].

It is conceivable that the disease connections related to cell size changes arise from changes in mitochondrial metabolism and the mevalonate pathway [10,46,67]. Thus, cell size effects on metabolism and cellular fitness may be useful for understanding certain aspects of these diseases. Consider the mevalonate pathway – cell size association. Inflammation is a signature of cellular injury and thus a potential indicator of reduced cellular fitness. Inflammation is also an important contributor to the pathogenesis of diabetes and many cancers [82-84]. Increased cell size, such as observed during compensatory growth of pancreatic beta-cells in insulin resistance [85], may be linked to reduction in mevalonate pathway activity [10,67], which induces an inflammatory response [86-88] (Fig. 5D). The mevalonate-pathway regulated inflammatory cytokines such as interleukin 1 beta, interleukin 6, and tumor necrosis factor alpha [88] could thus trigger an infiltration of inflammatory cells to tissues with abnormally large cells. It is also well established that cholesterol metabolites such as 25-hydroxy-cholesterol directly influence interleukin 1 beta expression [89] indicating an additional connection between cell size and inflammation at the tissue level. Another condition where cell size changes induce inflammation is obesity, which is associated with infiltration of inflammatory cells (mainly macrophages) into adipose tissue. The level of leukocyte infiltration is directly proportional to the adipocyte cell size [90] and both experimental and disease-related evidence suggests mevalonate pathway involvement [10,46,67,90].

Conclusions

We are still lacking a comprehensive view on how and why cell size is controlled. While progress in proliferating cell types has been good, size control mechanisms in non-proliferating cells remain less well understood. Despite this, there is a slowly increasing understanding that cell size affects cellular metabolism and fitness. This may indeed be the very reason why cell size control is required.

Changes in cell size and cell size variability are commonly observed in many disease conditions and during aging. Because current evidence suggests that cellular fitness is often, although not likely always, dependent on mitochondrial metabolic activity, it will be important to probe the biomedical significance of cell size control. We speculate that cell size changes during aging and in metabolic disease are not just correlations but manifestations of the same underlying mechanisms; problems in cell size control and associated metabolism. If so, then cell size could provide a novel framework for understanding some of the metabolic disease phenotypes and to suggest improved preventive and therapeutic strategies. The role of mevalonate pathway in inflammatory response is one example of this school of thinking. Altogether, cell size homeostasis may have emerged to function as a protective property that helps cells to maintain active metabolism and maximal fitness needed for organismal survival.

Future research should focus on examining the underlying molecular and biophysical mechanisms that result in size dependent changes of metabolism, and conversely, metabolic changes resulting in cell size changes. Also, our knowledge of which cellular functions are affected by cell size remains limited. Expanding these questions to non-proliferating cells and in vivo models will be challenging but critical for revealing how cell size control contributes to the organismal health and wellbeing. Polyploid organisms as well as genetically mosaic animals are likely to be the key models enabling the studies on size control mechanisms in multicellular organisms.

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Table 1. Common problems and pitfalls when studying cell size

	Example	Arising problem	Solution	
	Defining 'cell size'	Cell size has been measured as volume, area, cell length or an indirect measure, such as protein content.	Changes in cellular composition and shape can influence the results. Data normalization to different measures of cell size can significantly alter conclusions.	Consider what is the most appropriate measure of size for the question. Validate the results using an independent size measurement.
	<i>Model organism & cell type selection</i>	Cell size is commonly studied using <i>E. coli</i> , fission or budding yeast, or cultured mammalian cancer cells. Non-proliferating cells are rarely used.	There is unlikely to be a single universal solution for managing cell size, although similar biophysical limitations are likely to apply. Also, cultured cancer cells may have by-passed normal size control mechanisms.	Validate findings in different model organisms and use primary cells and in vivo models, when possible.
	<i>Cell size vs cell cycle</i>	Cells become larger at later stages of cell cycle, thus many cell cycle arrests appear to increase cell size if cell growth is unaffected.	Assumed cell size regulation or size dependent function may reflect a cell cycle effect.	Control for cell cycle changes in cell size studies.
	<i>Cell size vs growth</i>	Growth regulation can be studied independently of cell cycle by first blocking cell cycle and then perturbing size.	If normal cell cycle is inhibited, then cell size changes reflect growth regulation. This does not automatically mean that cell size would change when cell cycle is active.	If the goal is to find size, not growth, regulators, validate that the cell size effects are observed also when cell cycle is not perturbed.
	<i>Growth and nutrient conditions</i>	Cell size effects obtained through perturbing growth pathway may only be limited to conditions, where this pathway is active.	Nutrients and environmental conditions affect cell size, cell cycle and cell size perturbations.	Repeat experiments under various growth conditions.
	<i>Data normalization</i>	Larger cells typically grow faster in absolute terms (see Fig. 1). This is similar to saying that humans are stronger than ants and ignoring the size difference.	With many biological questions the data requires size normalization. Otherwise one may conclude that bigger is always better.	Consider the biological question and normalize data accordingly. Also, consider what is the correct size measure to which data should be normalized.
	<i>Cell size perturbations and size dependent functionality</i>	One may try to study cell size dependent functionalities by first making cells smaller or larger.	The size perturbation makes the data circumstantial. Cell size cannot be changed without possibly affecting the function of interest.	Utilize the pre-existing variability in cell sizes. This often requires single-cell approaches.

Figure legends

Figure 1. The need for cell size normalisation of growth rate

5 **A:** An example of a L1210 mouse leukemia cell growth rate through the interphase. These cells are rapidly growing with the total cell cycle time being ~11 h. Growth rate was obtained by continuously measuring the cells' buoyant mass using suspended microchannel resonators as in [31]. The obtained mass measurements (black dots) were used to plot a trend line (red line) of the growth rate as a function of time from cell division ("birth"). Without normalisation of growth to cell size/mass it would be easy to conclude that larger cell size is advantageous, as the absolute growth rate is higher.

10 **B:** Same data as panel A, except the growth rate was normalised to the cell mass at each time point. After normalisation, it is obvious that larger cell size does not mean more growth per unit of mass (i.e., growth is not exponential throughout the cell cycle), which often is the underlying biological question.

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Figure 2. Cellular fitness changes with cell size

20 Cellular fitness of many cell types displays a maximum at the intermediate size range in the population [46], indicating that there is an optimal size range. This range, where at least some of cellular functions are maximised, reflects also an optimal size range for progression through the cell cycle, as the largest and smallest cells within a population have reduced proliferative capacity in cultured cells [46]. Some of the potential mechanisms why too small or too large cells may display a fitness disadvantage and limitations to the metabolic rate are highlighted below.

25 Figure 3. Hepatocyte polyploidisation and effects of Cdk1 on hepatocyte size and metabolism

30 **A:** Hepatic polyploidy is common during liver development and results in increased cell size. However, cellular damage also induces hepatocyte polyploidy, suggesting that polyploidisation is also used for cellular adaptation. It is not clear whether this is a two-way regulation, i.e., ploidy increasing oxidative stress and metabolic overloading.

35 **B:** Liver regeneration in the presence and absence of Cdk1. Before partial hepatectomy, hepatocytes display low cellular turnover, while maintaining high metabolic rates. At this point normal diploid hepatocytes display a balance between glycolytic rate and mitochondrial oxidative phosphorylation (OxPhos). During liver regeneration, when hepatocytes grow in size and prepare for division, mitochondrial OxPhos is reduced and glycolysis is enhanced. Mitochondrial OxPhos and glycolysis return to their normal levels after cell division. In the Cdk1^{Liv^{-/-}} hepatocyte regeneration results in increase in ploidy and cell size, while mitochondrial OxPhos is further reduced and glycolysis increased after partial hepatectomy. This suggests that

40

the changes in cellular energy metabolism are dependent on cell size and/or ploidy rather than on regeneration or proliferation.

5 **C:** Hematoxylin&Eosin stained histological sections before and after partial hepatectomy. Liver regeneration in the absence of Cdk1 results in massive increases in cell size as evidenced by the highly enlarged hepatocyte nuclei in the Cdk1^{Liv-/-} after partial hepatectomy (PH).

Figure 4. Cell size and shape dependent biophysical limitations to growth and functionality

10 **A:** Cell size and shape affect surface-to-volume (SV) ratio and intracellular distances. As many key features of cells metabolism, like protein synthesis and transcription, take typically place close to the center of the cell, a spherical shape in large cells may generate intracellular metabolite and nutrient gradients. While oxygen levels are higher close to the cell surface [72], the gradient directionality might be reversed for
15 “waste” products such as lactate. Thus, complex non-spherical shapes may provide a fitness advantage to large cells. Large spherical cells may display reduced metabolic fitness compared to cells having elongate or other more elaborate shapes. The upper right corner displays plasma membrane stained B-cells, which have a spherical morphology typical for many small animal cells.

20 **B:** Effect of intracellular distances to metabolism. Amino acids, glucose and oxygen travel via diffusion whereas organelles are moved using active transport. Travel time with diffusion in a three dimensional space will increase exponentially with the distance, i.e., travel time is proportional to (\propto) the square of the distance. Instead,
25 travel time with active, directional transport will increase linearly with the distance (see [73] for a review). The total distance of a metabolic route relying on diffusion can be surprisingly long. For example, glucose must travel much more than just the radius of the cell when being used as an energy source for growth. The first metabolic enzyme in glycolysis, hexokinase (HK), is typically bound to mitochondria [91].
30 From there glucose-6-phosphate (glucose-6-P) has to travel to each other enzyme in glycolysis, and then back to mitochondria for ATP production. The ATP molecules need to reach ribosomes or other sites of energy consumption before the original energy stored in glucose is converted in to biomass and growth. Metabolism is therefore strongly affected by intracellular distances. Appropriate intracellular
35 localization of cellular components may help cells grow larger.

C: Log-log plot of travel times and distances for active directional transport (axonal transport) and diffusion of different sized molecules in cytoplasm illustrates the challenge for diffusion-mediated process in large cells [73,92,93].

40 **D:** Log-log plot of typical SV ratios and volumes of different cell types ([94] and references within). The blue line displays the SV ratio for a perfect sphere. The SV ratios areas at which most bacteria, yeast and human cells occupy are highlighted in green, yellow and red, respectively. The vast differences in SV ratios are likely to affect metabolic activity and functionality. Note that, for example, the human embryo

has very low SV ratio, but this is increases approximately 22% with every cell division in the blastomere [95], which may be critical to support growth in the early embryo.

5 **Figure 5. The mevalonate pathway affects cell size, metabolism and immune response**

10 **A:** Mevalonate pathway inhibition using cholesterol lowering drugs statins increases cell size in human and *Drosophila* cells as analysed by flow cytometry (see ref [10]). Note that statins inhibit an early part in the mevalonate pathway, thus reducing the levels of numerous lipophilic metabolites required for normal mitochondrial function, cell signaling, autophagy and plasma membrane synthesis [10,46,79,86,87]. It is worth pointing out that high concentrations of statins can severe toxicity, which reduces cell size, and different cell types have vastly different sensitivity to statins [10].

15 **B:** Statins influence mitochondrial dynamics in human cells. Jurkat cell mitochondria (green) were imaged after mevalonate pathway inhibition with statins. Plasma membrane is shown in red. Image from ref [46], reproduced under CC BY 4.0 license.

20 **C:** The mevalonate pathway affects cell size dependent metabolism as exemplified by statin effects on two parameters of oxygen consumption, maximal and ATP producing respiration. Flow cytometry based cell size distributions are shown below as a reference. Data is from ref [46].

25 **D:** A potential mechanism for cell size dependent induction of inflammation. Abnormally large cell size is associated with reduced mevalonate pathway activity [10,67]. The mevalonate pathway inhibition induces expression of inflammatory cytokines such as interleukin 1 beta (IL-1 β) [86,87]. These cytokines activate leukocytes to infiltrate into tissues displaying loss of size homeostasis (hepatocytes in this case), resulting in tissue inflammation. As inflammation can further downregulate the mevalonate pathway [86,87], this may induce a feedback causing further increase in size and enhanced inflammation.

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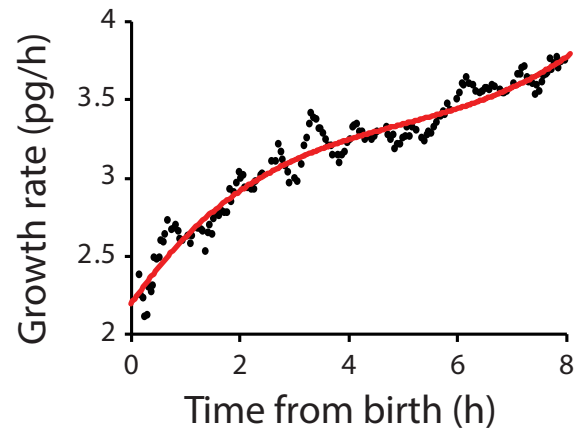
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Fig 1. Miettinen et al.

A) Interphase growth rate
without size normalisation



B) Interphase growth rate
with size normalisation

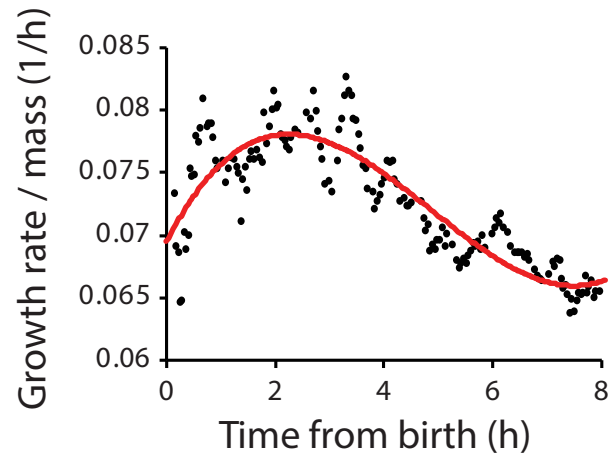
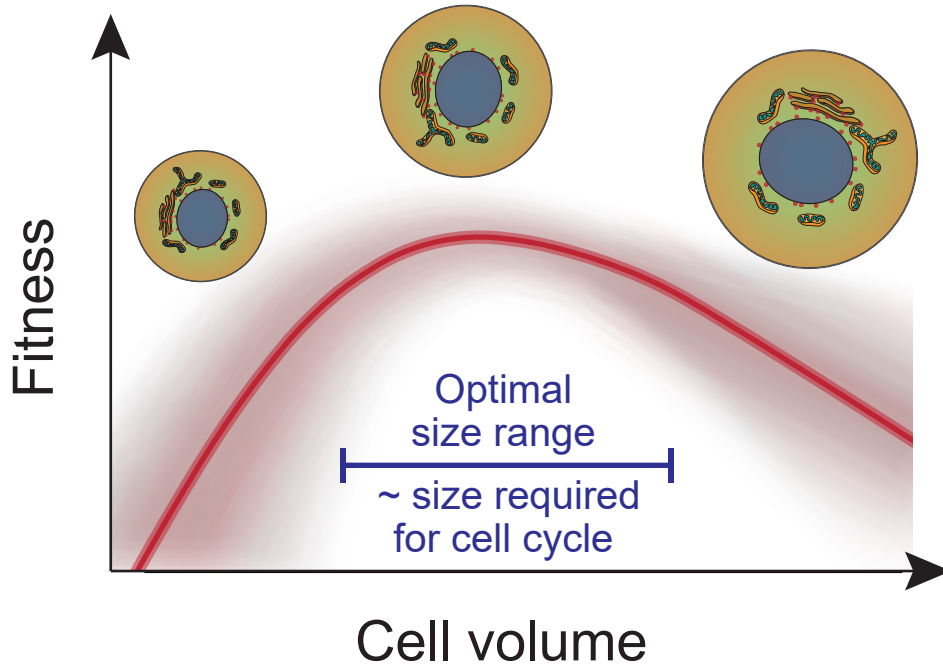


Fig 2. Miettinen et al.



TOO SMALL CELLS

- Sensitivity to apoptosis
- Limited biosynthetic capacity
- High cost for maintaining ionic gradients

TOO LARGE CELLS

- Membrane and cytoskeletal integrity
- Increasing intracellular distances
- Reduced surface area for nutrient exchange

→ *Limitations to metabolic rate, reduced fitness*

Fig 3. Miettinen et al.

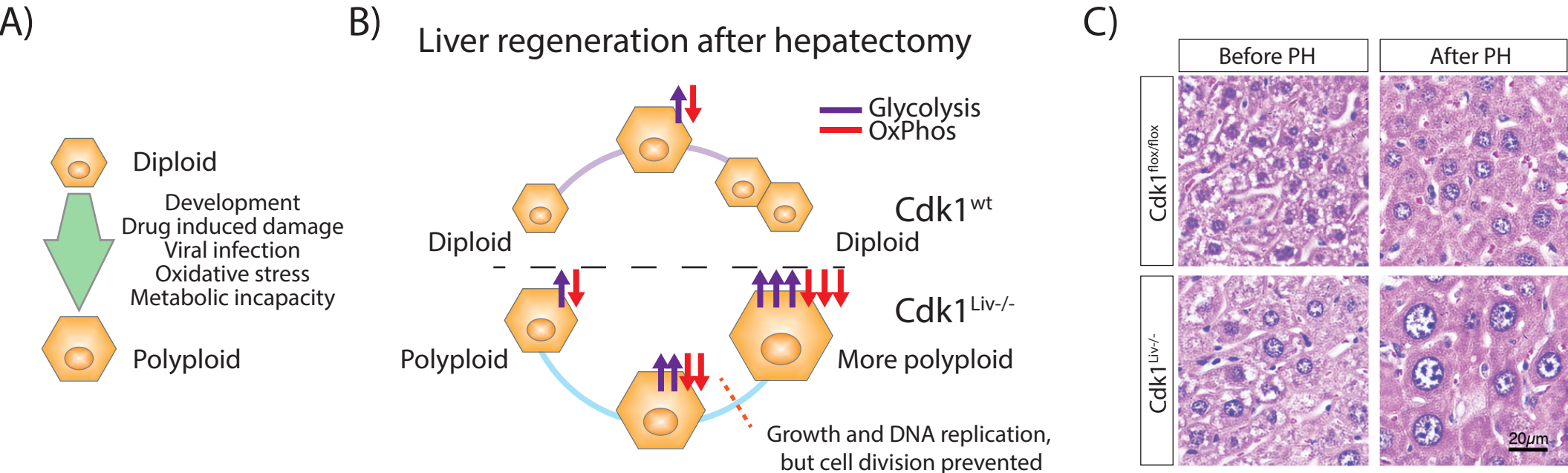


Fig 4. Miettinen et al.

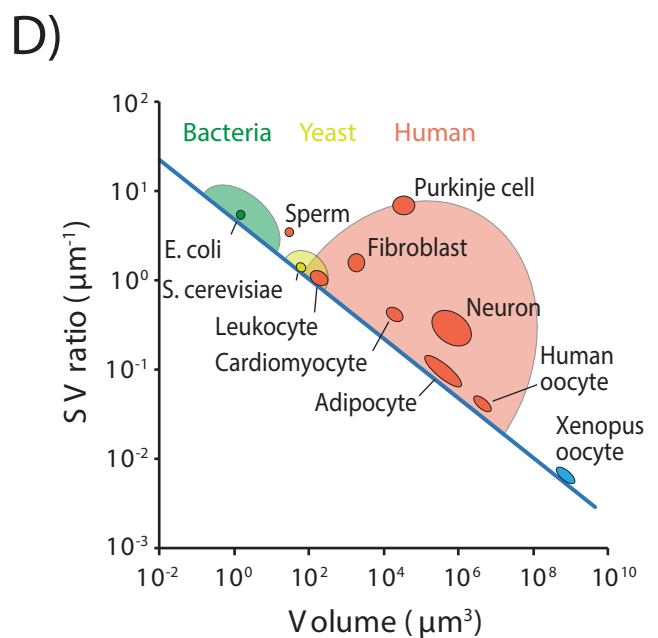
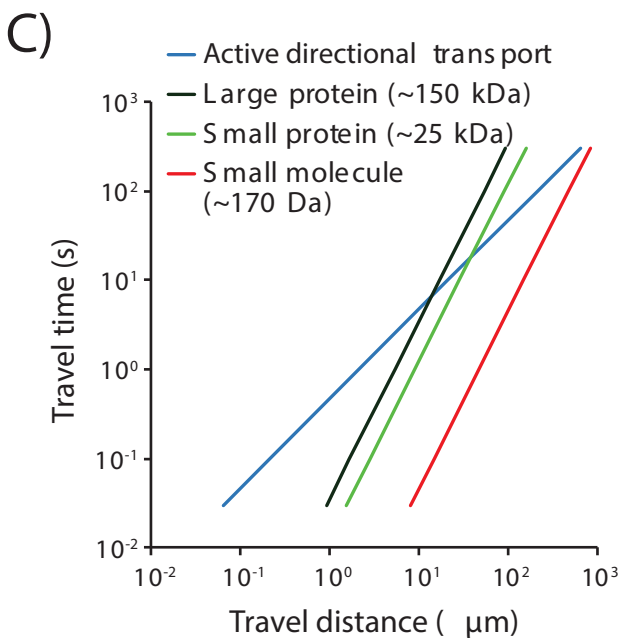
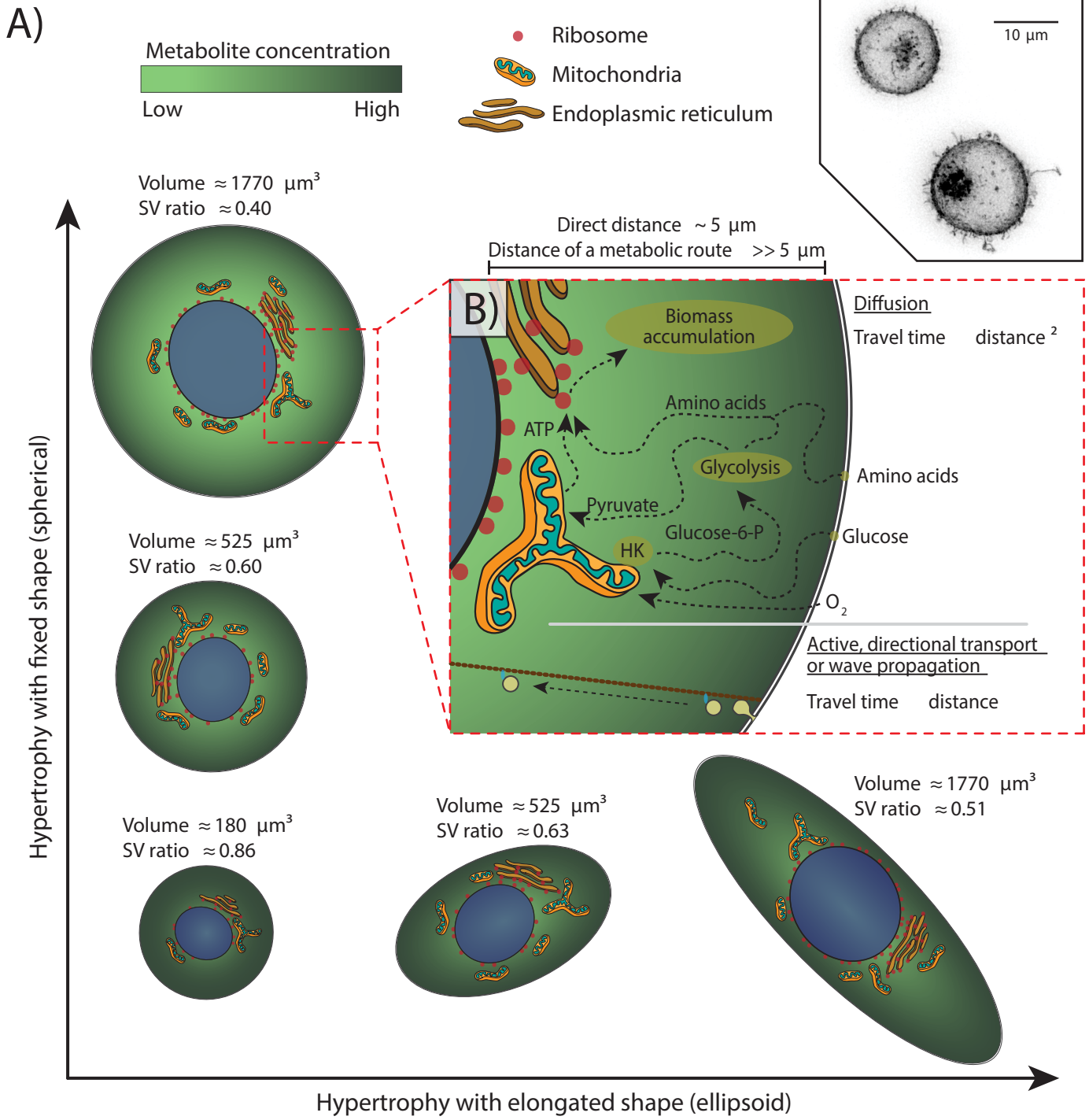


Fig 5. Miettinen et al.

