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Cell Biology: The Health Hazards of Super-Sizing

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greater skill retained following interleaved, as opposed to a single block, of practice, when skill is acquired 'online' during practice [17]. However, skill may not ever be acquired during practice: it may always be acquired following practice. Practice may trigger a set of offline processes which, seconds later, lead to the acquisition of skill. Rather than occurring during practice, skill learning occurs several seconds after practice. Learning lags practice and so occurs offline after practice has ceased. This lag may be the inevitable consequence of implementing learning within a biological substrate, dependent upon time-consuming processes such as protein synthesis [18].

Until now lengthy blocks of practice have perhaps hidden this short temporal delay between practice and subsequent skill acquisition. The skill is acquired after practice, but, more practice is being performed, and so the skill is assumed (misattributed) to come from that subsequent practice. Only when practice is massively extended, and there is no subsequent need for offline processing — with prolonged practice a skill memory does not require subsequent offline stabilisation — does it become apparent that offline processing is occurring latently during practice [19]. By elegantly distinguishing between the contributions of practice and rest to skill acquisition, Bönstrup *et al.* [6] have challenged the idea that skill is acquired during practice, and shown instead that much, if not all, our skill is acquired offline during rest.

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Cell Biology: The Health Hazards of Super-Sizing

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Cells typically occupy a narrow range of sizes according to their type. A new study reveals that cells grown to gigantic proportions fail to synthesize sufficient macromolecules, resulting in cytoplasm dilution and a loss of fitness reminiscent of old cells.

> "*Why am I soft in the middle when the rest of my life is so hard?*"
>
> – Paul Simon

Size is a fundamental feature of biological systems that is tightly controlled at the cellular level. How cells coordinate their growth to maintain specific size-related features, such as surface area and volume, while at the same balancing their macromolecular content, is poorly understood. What are the determinants of cell-size parameters? What happens when normal size relationships are perturbed? A recent study published in *Cell* by Neurohr *et al.* [1] highlights the

many consequences of increased cell size on cellular functions and raises several challenging questions regarding the coordination of cell size in terms of mass, volume, and surface area.

To generate large cell sizes, Neurohr *et al.* [1] cultured budding yeast under conditions that allowed cell growth but blocked progression through the cell division cycle. These cells reached a volume that was six times larger than normal cycling cells and displayed an array of interesting defects. The authors confirmed that the effects they observed were specific to the large cell volume and not to delayed cell-cycle progression by comparing the same cells grown in glucose-poor medium, where they maintained small volumes.

The authors first monitored the consequences of large cell volume on the recovery from cell-cycle arrest. Large cells, but not small cells, showed a reduction in proliferation rate and a delay in each stage of the cell cycle, taking longer to replicate their DNA and undergo division. Interestingly, gene expression of key cell-cycle regulators, including the G1 cyclin *CLN2* and the mitotic cyclin *CLB2*, was impaired, prompting the authors to investigate whether a reduced ability to turn on genes was a general consequence of an abnormally large cell volume. In large cells, gene induction of the galactose-inducible *GAL1* gene was reduced due to continued *GAL1* promoter repression, despite galactose addition to the culture medium. Pheromone induction was also altered in large cells by a mechanism that involved defects in the MAPK signaling cascade. Thus, several gene-induction pathways dependent on either extracellular or intracellular mechanisms are altered in large cells.

What might be the basis of the defects caused by large cell size? In further characterizing the physiology of large cells, the authors quantified the rate of increase of RNA and protein levels as cells expanded. They found that cell volume increased faster than protein and RNA amounts, ultimately causing a dilution of cell cytoplasm. After 6 hours of cell-cycle arrest, cellular density decreased by 36%. The authors then investigated several potential explanations for such an intriguing decoupling of the rate of protein and RNA synthesis from that of volume increase.

One explanation is that the biosynthesis machinery becomes compromised in large cells. RNA sequencing and mass spectrometry analysis revealed that general transcription and translation factors were under-represented in large cells. Also, large cells showed evidence of the activation of the well-characterized environmental stress response (ESR). This response is known to cause a downregulation of translation, and, to a lesser extent, transcription. Importantly, the authors showed that the ESR also leads to cytoplasm dilution in small cells. Thus, the ESR contributes to the decoupling between cell volume growth and RNA and protein levels.

To test whether gene copy number could become limiting for biosynthesis and thus contribute to the decoupling with volume growth, the authors used an elegant approach whereby they controlled the timing of arrest in the cell cycle to generate haploid and diploid cells of similar cell volumes from the same initial population. Strikingly, defects in galactose- or pheromone-dependent gene induction were reproduced in diploid cells, but at larger volumes than for haploid cells. Furthermore, protein concentration in the cytoplasm decreased at larger volumes in diploid cells compared with haploid cells. Thus, gene copy number is involved in setting the maximum size at which the rate of protein synthesis can keep up with an increase in cell volume.

In the last series of experiments, the authors turned to the link between cell size and aging, given that old cells are enlarged and have a reduced lifespan. They observed that young yeast cells that have grown large possess many of the phenotypic characteristics of old cells and, remarkably, undergo fewer replicative divisions than small cells, suggesting a link between large cell size and lifespan. The increased size of aging cells is an evolutionarily conserved phenomenon and is accompanied by a terminal cell-cycle arrest in mammalian cells termed senescence. Experiments with human fibroblasts, a much less tractable system than yeast, also indicate connections between excessive cell growth, decreased macromolecular crowding, and cellular senescence.

Altogether, the work of Neurohr *et al.* [1] has produced a wealth of experimental evidence that cell size has profound effects on cellular physiology. This work raises a number of important and interesting questions that will stimulate several areas of research (Figure 1).

Firstly, how is cell density regulated during growth? The maintenance of cellular density is crucial for proper cellular function, and ribosomal crowding, for example, has been shown to regulate phase transitions in human cells [2]. However, how distinct size variables such as mass and volume are coupled to maintain a constant cell density is not understood. A recent preprint [3] has shown that, in the fission yeast *Schizosaccharomyces pombe*, growth under conditions in which volume expansion, but not protein synthesis, was prevented resulted in very high cytoplasmic protein concentrations. When cell-wall expansion was allowed again, cells underwent a period of fast volume growth and rapidly diluted the cytoplasm back to its normal protein concentration. Thus, compensatory mechanisms exist when cellular densities are higher than normal.

What happens in the opposite scenario when volume, not protein amount, keeps expanding? Here, Neurohr *et al.* [1] have established an experimental system that uncouples growth from protein synthesis rate at yeast cell volumes greater than 200 femtoliters. One could hypothesize two regulatory mechanisms to correct low intracellular density: downregulation of surface area addition, or upregulation of protein synthesis rate. Previous studies have shown that large budding yeast cells tend to downregulate the expression of proteins associated with surface area structures [4], but this observation is not reproduced in the dataset in the new study, perhaps due to a difference in the timescales at which the measurements were made. Theoretically, if the production of rate of cell surface area proteins is the same as that of cytoplasmic proteins, an imbalance between cell mass and cell volume would occur because, as the cell grows, its surface area to volume ratio decreases. An excess of cell-surface proteins would be produced relative to cytoplasmic proteins, since the surface area is increasing with the radius squared, while

cell volume increases with the radius cubed. Unlike the rate of protein synthesis, Neurohr *et al.* [1] found that lipid and carbohydrate amounts were not affected in large cells, supporting the idea that surface area could expand more quickly than volume, leading to an imbalance and reduced cellular density. Overall, although much is known about the regulation of cell surface area in yeast [5], how it is coupled to biomass synthesis remains unclear.

Secondly, what is the link between ploidy and protein synthesis rate? A possible hypothesis supported by recent experimental and theoretical work [6,7] is that, below a given protein:DNA ratio, gene copies become limiting for transcription, thus giving rise to a linear protein synthesis rate. The observation by Neurohr *et al.* [1] that the scaling between transcript levels and volume is lost earlier than the scaling of protein production with cellular volume is in line with this hypothesis (see Figure 4A,B and D,E in [1]).

Furthermore, the delay in size-induced defects of gene induction in diploid compared with haploid cells strongly suggests a contribution of increased ploidy, and therefore increased transcriptional capacity, in rescuing the phenotypes. However, a deeper understanding of how ploidy may become limiting for transcription and mass increase will require direct measurements of the protein synthesis rate and instantaneous transcription rate at different cell sizes, perhaps using recent sophisticated techniques for single cells [8,9].

Other experimental evidence suggests that a general increase in protein:DNA ratio is not the sole explanation for the decoupling between protein synthesis rate and volume growth. Neurohr *et al.* [1] also document a specific downregulation of transcription and translation factors in large cells and an activation of the ESR pathway. Protein concentrations affect their reaction kinetics and compelling examples of how cell-cycle transitions are driven by specific concentrations of regulator have been reported [10]. Protein synthesis rate also directly relates to ribosome concentration in the cell [11–13]. What causes the downregulation of transcription and the activation of the ESR pathway in large yeast cells remains unclear, but the data suggest that regulatory pathways in the cell might be directly affected by large volume or by cytoplasmic dilution.

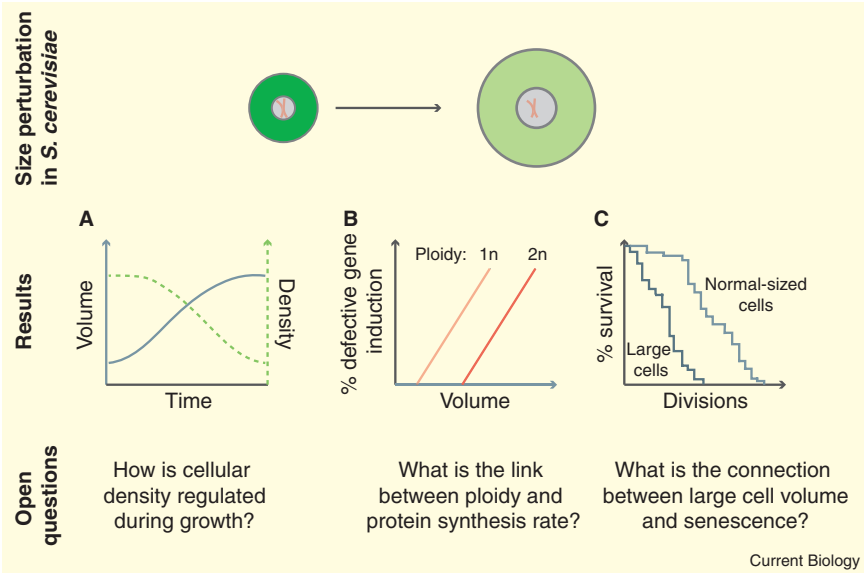

Figure 1. Increasing cell volume in budding yeast: consequences and open questions.
By blocking cell-cycle progression but not growth, Neurohr *et al.* [1] make several intriguing observations. (A) Cell volume expansion outpaces an increase in cell mass because protein synthesis rate reaches a plateau. This leads to a progressive dilution of cell cytoplasm and a decrease in cellular density. (B) Many cellular functions, such as gene induction, are impaired. The defects occur at larger volumes for diploid cells, thus suggesting a role for transcriptional capacity in enabling large cell volumes. (C) Young large cells show many common features with senescent cells, raising the possibility that senescence results from large cell volumes, perhaps as a consequence of cytoplasm dilution.

Finally, what is the connection between large cell volume and senescence? Although Neurohr *et al.* [1] do not directly demonstrate that cytoplasm dilution or decreased protein:DNA ratio directly causes cellular senescence, they show that large young cells have a decreased survival rate compared with normal-sized young cells. Moreover, they show that molecular crowding decreases as the size of human fibroblasts increases under cell-cycle arrest and that these cells fail to resume proliferation following release from arrest. Further experiments — for example, comparing the fitness of small compared with large diluted cells of different ploidies — will help to resolve this point.

These final results from Neurohr *et al.* [1] raise an interesting perspective: that altered reaction kinetics in cells due to a change in density can lead to senescence. Altogether, these observations heighten our need to understand how cell size and mass are determined and coordinated in the first place.

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Learning: Complexities of Habituation in Escaping Zebrafish Larvae

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Animals decrease responses to repeating stimuli through habituation. New research has revealed independent tuning of multiple parameters of zebrafish escape behavior during habituation.

Behavioral habituation is perhaps the simplest and most common form of learning [1]. Habituation happens when a repeating stimulus reduces subsequent responses. The ticking of a new clock might disrupt our sleep for a few hours, or even a couple of nights in a row, but will soon fade until we hardly hear it anymore. Similarly, even the grimmest trick of a fake skeleton cannot scare us more than a handful of times, with our fearful reflexes largely mute by the end of a Halloween party. How does the brain achieve this remarkable feat of adjusting its response to one and the same input so profoundly? In this issue of *Current Biology*, Randlett *et al.* [2] report exciting new data on the regulation of behavioral responses during habituation in larval zebrafish.

Habituation research is at least 100 years old and has covered the tree of life from unicellular organisms to invertebrates and humans [3]. Until the 1960s, habituation research focused on psychophysics and distilled parameters of sensory stimuli that affect habituation. As a rule, stimuli cause more rapid habituation when they occur more frequently, stronger stimuli take longer to habituate than weaker ones, and responses recover when the stimulus ceases [1]. Since then, investigations of habituation have produced deep insights into its molecular, neural and synaptic implementation. Pioneering studies in the sea slug *Aplysia californica* by Eric Kandel and colleagues traced the motor circuit underlying the gill withdrawal reflex to touch and mapped key molecules that alter synaptic properties within this circuit and habituate the reflex upon subsequent prodding [4].

Randlett *et al.* [2] focus on habituation in the context of escape responses to sudden changes in illumination. The authors show that escape habituation involves independent adjustments of multiple, fine-grained behavior parameters [2]. These results illuminate an aspect of habituation that had been relatively unexplored. In part due to a lack of precise tools for behavioral measurements, most studies have classified behavioral habituation either as a reduced probability of a binary response or as reduced response intensity using a single continuous parameter (Figure 1B). To overcome these limitations, Randlett *et al.* [2] devised an apparatus to study habituation simultaneously in 600 larval zebrafish with high temporal and spatial precision to extract detailed kinetic information about behavior. By repeatedly switching off the lights they induced ‘dark flash’ responses, a type of escape in which larvae reorient and swim away from their initial position (Figure 1A).

The authors quantified eight response parameters, such as probability of response, latency, and bend amplitude (Figure 1C). Dark flashes were presented in five blocks of 60 dark flashes, with one-minute inter-stimulus interval and one-hour inter-block interval. This protocol, which initially triggered robust escapes across the population, induced long-term habituation lasting for longer than 24 hours (Figure 1C). To examine if learning affected specific parameters, the authors used the statistical power of their apparatus and analysed escapes in thousands of individual larvae across experiments, each responding to hundreds of stimuli.