UNIVERSITY OF CALIFORNIA

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RECENT ADVANCES IN UNDERSTANDING CONTROL OF CELL GROWTH AND SIZE IN MAMMALIAN CELLS

A thesis submitted in partial satisfaction of the
requirements for the degree of

MASTER OF SCIENCE

in

MOLECULAR, CELLULAR,
DEVELOPMENTAL BIOLOGY

by

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March 2024

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Abstract:

Technological and computational advances in analysis of cell size have led to new insights into mammalian size control. These new techniques have allowed high-resolution, single-cell measurement of size in large numbers of proliferating cells, which allowed testing of models of cell size control. Here, I will describe the techniques used, and put the data generated into context for the established models of size control that are currently under debate. After a comprehensive study of the literature these techniques support an adder model of growth control in mammalian cells, in which there is a constant amount of growth generated per cell cycle, regardless of starting cell size.

Introduction:

Cell cycle progression is dependent upon cell growth, which ensures that cells maintain a consistent size. Tight control of size is important for proper development and regulation of organ function and maintenance in metazoans. Careful control of cell growth and coordination with cell cycle progression is essential for viability and maintenance of specific cell size. Surprisingly, little is known about the fundamental processes that coordinate and regulate cell size and growth. The mechanisms that control how much growth occurs during the cell cycle are largely unknown.

Variability in cell size can be caused by different factors, such as modulation of growth rate, duration of the cell cycle, asymmetric division, or biochemical stochasticity during the lifespan of a dividing cell. A large heterogeneity in size and defect in nuclear to cytoplasmic ratio are a nearly universal feature in cancer. Thus, a better understanding of the pathways and mechanisms that control cell growth can be a novel avenue for selectively targeting cancerous cells. Recent work in mammalian cells suggests two separate models of size control. The first is the “sizer”, where a cell grows until reaching a specific size threshold allowing entrance into the cell cycle. Originally the sizer model was suggested as the model of growth control in mammalian cells, based on population-level measurements (7). This model suggests that size control exists solely during G1 and is dependent upon cell size at birth. The second model is the “adder”, originally suggested as the size control mechanism in bacteria (8), where cells add a constant, fixed amount of growth prior to division regardless of their starting size. This model implies that cell growth is measured to ensure that the same amount of growth occurs during each cell cycle.

Models of cell size control

To date, there is conflicting evidence supporting each model of cell size control. The sizer model proposes that cells must grow until they reach a specific size threshold in G1 triggering entry to the cell cycle (Figure 1). If there is a specific size at which cells enter the cell cycle, upon transition to S-phase, all cells will reach the same size going into subsequent cell cycle stages. This means cells that start smaller than the average size will grow more during G1, and cells that are larger will grow less. If cells grow to a certain size regardless of starting size, there will be no correlation between size at birth and size at cell cycle entry (G1/S transition). Lastly, if cell size homeostasis is maintained solely in G1, during S/G2/M cell size should maintain a constant state of growth and stable variability of size. The sizer model posits that cells measure their size when making decisions about cell cycle progression.

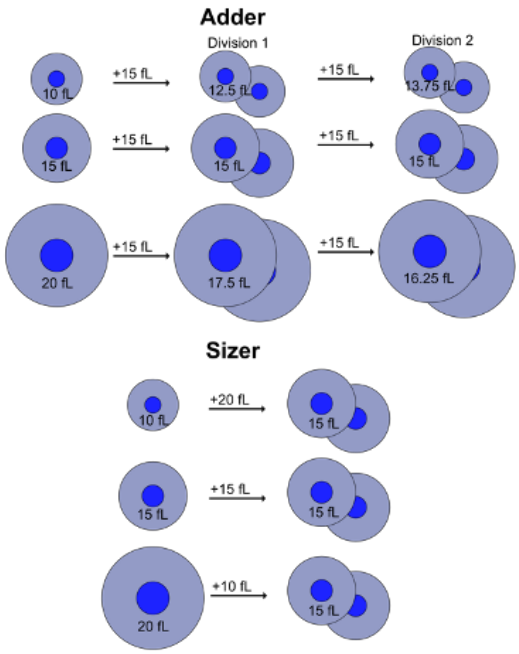

Figure 1: Schematic showing how adder and sizer models could allow cells of below- and above-average size to reach homeostatic size.

The adder model suggests that a constant amount of growth is added per cell cycle. This means that regardless of starting size each cell will accumulate the same amount of growth per cell cycle. Adding a fixed amount of growth each cell cycle leads to an iterative correction in size, allowing cells

to approach an asymptotic size over multiple cell cycles (Figure 1). If cells grow a constant amount, there should be no correlation between size at birth and growth during the cell cycle, but there will be a direct correlation between size at birth and size at division. The adder model suggests that cells measure the extent of growth, rather than size.

With the complexity of mammalian cell machinery, variability in shape and size between cell types, and coordination required of metazoan cells, it is possible that these simplified models are not sufficient to explain size control. A combination of the two models is possible; different cell types, cell cycle phases, or cells at the extremes of a population's cell size distribution could all use a different form of size control.

Evidence for models of size control

Technically, measuring cell size is challenging in mammalian cells. Adherent cell lines in culture are difficult to measure the size of by microscopy due to irregular shape and height in the z-axis. Simultaneously measuring cell cycle progression and cell size can be difficult; achieving accurate measurements of individual cells requires high resolution and throughput. To better understand which model best describes the control mechanisms in mammalian cells, various methods have been developed recently to measure growth, such as fluorescent dye exclusion (FXm) (1), computationally-enhanced Quantitative Phase Microscopy (ceQPM) (2), or Channel-Assisted

cell reshaping restricting growth to one axis (3). The methods use volume, mass, or area extended as metrics of growth.

Both sizer and adder models can fit various growth rates/growth speeds depending on the potential regulation of phase or total cell cycle duration. If the duration of growth was changed in coordination with the growth rate or growth speed, either model could fit. If a smaller cell has a lower growth rate, it need only increase the duration of growth to hit its threshold size or achieve the specified amount of growth for one cell cycle.

Evidence for the sizer model & potential mechanisms

The sizer model dictates that a threshold size must be reached for a cell to enter the cell cycle. This means that small cells will grow more during G1, and large cells will grow less, to reach the size threshold necessary to commit to a round of cell division and enter S-phase. If this is the case, size at birth will be directly proportional to volume accumulated through G1. Currently, the evidence that exists in support of the sizer is only within the G1 phase and is mercurial.

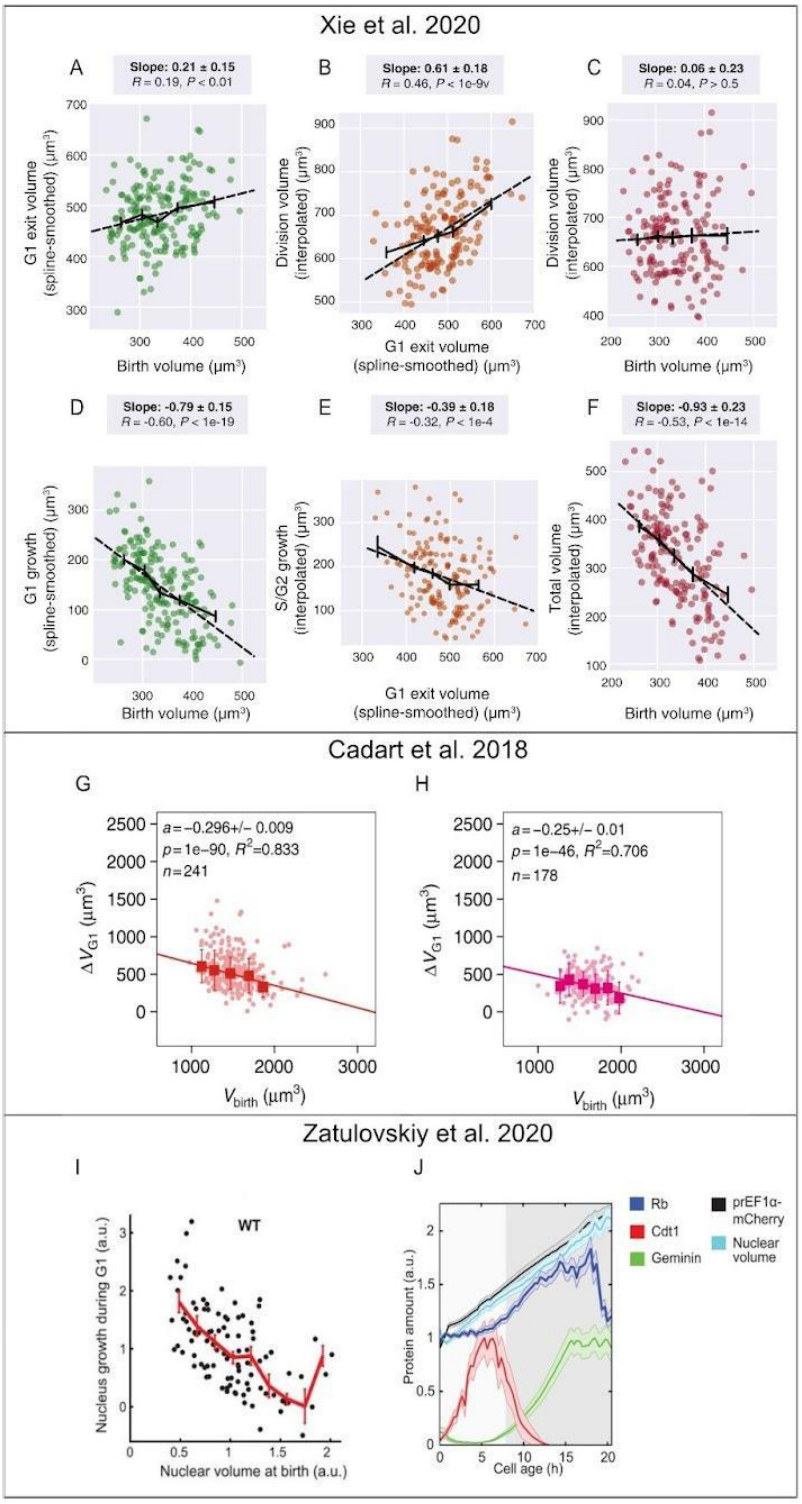

Figure 2: Measurements of single cells showing sizer behavior and proposed mechanism of action. A-F) Xie et al. 2020, figure D-I, The cell birth volume is plotted against the G1 exit volume (A). The G1 exit volume is plotted against the volume at division (B). The birth volume is plotted against the volume at division (C). (D–F) The amount of volume grown during the respective phases is plotted against the cell volume at the beginning of the indicated phase (D and E) or the entire cell cycle (F). G,H) Cadart et al. 2018, figure 4 e,f, volume at birth vs. volume accumulated during G1 for HT29-hgem (G) and HeLa (H). I,J) Zatulovskiy et al. 2020, figure 4H, nuclear volume at birth vs nuclear growth during G1 (I), figure 1K, fluorescence (a.u.) of various cell cycle markers, nuclear volume, total protein, shaded area represents average end of G1 (J).

The strongest evidence of sizer-like behavior was seen by Xie and Skotheim where mouse epidermal cells were measured, in vivo, over one week (6). Mice were tattooed to iteratively locate, image, and measure cells. Cell cycle progression was tracked by a FUCCI G1 mKO2-hCdt1 reporter. While a unique approach to gather in vivo data about cell size, these measurements require orthogonal validation of their accuracy. The authors mention that G1 reporter expression was used to track G1 progression, yet it was inconsistent across different time points. G1 end was marked by indistinguishable reporter fluorescence from background signal. This would cause difficulty in measuring the amount of growth through G1, as its duration might not be accurately defined. A slight correlation between birth volume and volume at G1 exit was observed (6)(Figure 2A) and a large correlation was seen between G1 exit volume and volume at mitosis (6)(Figure 2B), supporting the adder. Importantly, no correlation was seen between birth volume and division volume, in direct support of the sizer model (6)(Figure 2C). Similarly supporting the sizer model was a direct correlation between volume at birth to volume accumulated during G1 (6)(Figure 2D) and volume at G1 exit and volume accumulated during S/G2 (6)(Figure 2E). This was also seen when looking at the birth volume versus the volume accumulated through the entire cell cycle (6)(Figure 2F). Interestingly, a wide size distribution at G1 exit and division volume is seen, arguing against a size threshold (6)(Figure 2A-C, E).

Cadart et al. used FXm in tissue culture cells to show a slight negative correlation between volume at birth and change in volume during G1, suggesting smaller cells grow slightly more than larger cells (1)(Figure 2G,H). Although the slight proportionality between size at birth and volume accumulated during G1 was observed, there is a large variation in size at the G1/S checkpoint. When plotted against each other, the volume at birth versus volume at G1/S (or volume at mitosis) more closely fit an adder model (1)(Figure 3F). Because the correlation doesn't fit the adder perfectly, yet was closer to the adder than the predicted correlation of the sizer or timer, the observed variation led the authors to dub the term "near-adder".

Similar results were reported by Zatulovskiy et al. who measured nuclear growth as an analog to cell growth. During G1 they saw total nuclear growth scaled with nuclear volume at birth in HMEC-tert cells (4)(Figure 2I). An important caveat to these data was it could only be observed under the condition where these cells were treated with Palbociclib (a CDK4/6 inhibitor). The CDK4/6-Cyclin D complex is active during G1, and inhibition of CDK4/6-Cyclin D causes a dramatic increase in G1 duration (2,4,5). Inhibiting the cell cycle makes measuring size control difficult, as the two are inherently linked and cells continue to grow during the cell cycle arrest.

Although direct evidence in support of the sizer model is sparse, biochemical mechanisms have been suggested in support of it. Zatulovskiy et al. suggest a dilution model, as was suggested for budding yeast *Saccharomyces cerevisiae* (4). This group suggests Rb is the "functional

homolog" to Whi5 in *S. cerevisiae*. Rb is a known inhibitor of E2F transcription factors, responsible for production of the G1/S cyclin, Cyclin E. The dilution model suggests a protein is at a constant amount, and as the size of the cell increases, the concentration of an inhibitor of cell cycle entry is diluted. Once the cell reaches a certain size, the protein is diluted enough to trigger entry into the cell cycle. Zatulovskiy et al. report Rb concentration is diluted during G1 around 20% at the G1/S checkpoint, relative to total protein and nuclear size (4)(Figure 2J). Rb seems to be an important player in cell size regulation as KO in mice causes decrease in cell size (4) and an increase in cell size CV. However, this could be due to its role in cell cycle regulation. Whether this is a robust enough signal to initiate a significant event such as cell cycle entry remains unclear at this time. Progression into the cell cycle needs to be carefully controlled. With such a small dilution factor, it is unclear how this mechanism could provide robust control of cell size at cell cycle entry.

Evidence for the adder model

Under the adder model, cells should add a constant unit of growth during each cell cycle, regardless of starting size, leading to a linear correlation between size at birth and size at mitosis, and there is no correlation between total change in size and size at birth. Multiple groups have found direct evidence of a correlation between size at birth, and size at either G1/S transition (3) or mitosis (1,2). Varsano et al. have shown through Channel-Assisted Cell Reshaping that there is a direct correlation between

cell size at birth and size at the G1/S transition (3)(Figure 3B). If the adder is correct and growth accumulation is a constant amount for cells of all starting sizes, cells will show variability in size at the G1/S checkpoint. Opposing the sizer, where all cells would be the same size at G1/S, variability of size at G1/S has been reported by various groups (1,2,3,4)(Figure 3B,C). Zatulovskiy et al. have shown, by measurement of nuclear volume as an analog to size, that there is a large distribution of cell size at the G1/S transition (4)(Figure 3C). Although this excludes S/G2/M, many suggest that cell size control occurs exclusively during G1 to ensure that a sufficient amount of growth has occurred (or in the case of the sizer, the cell has reached a particular size) allowing entry to the cell cycle.

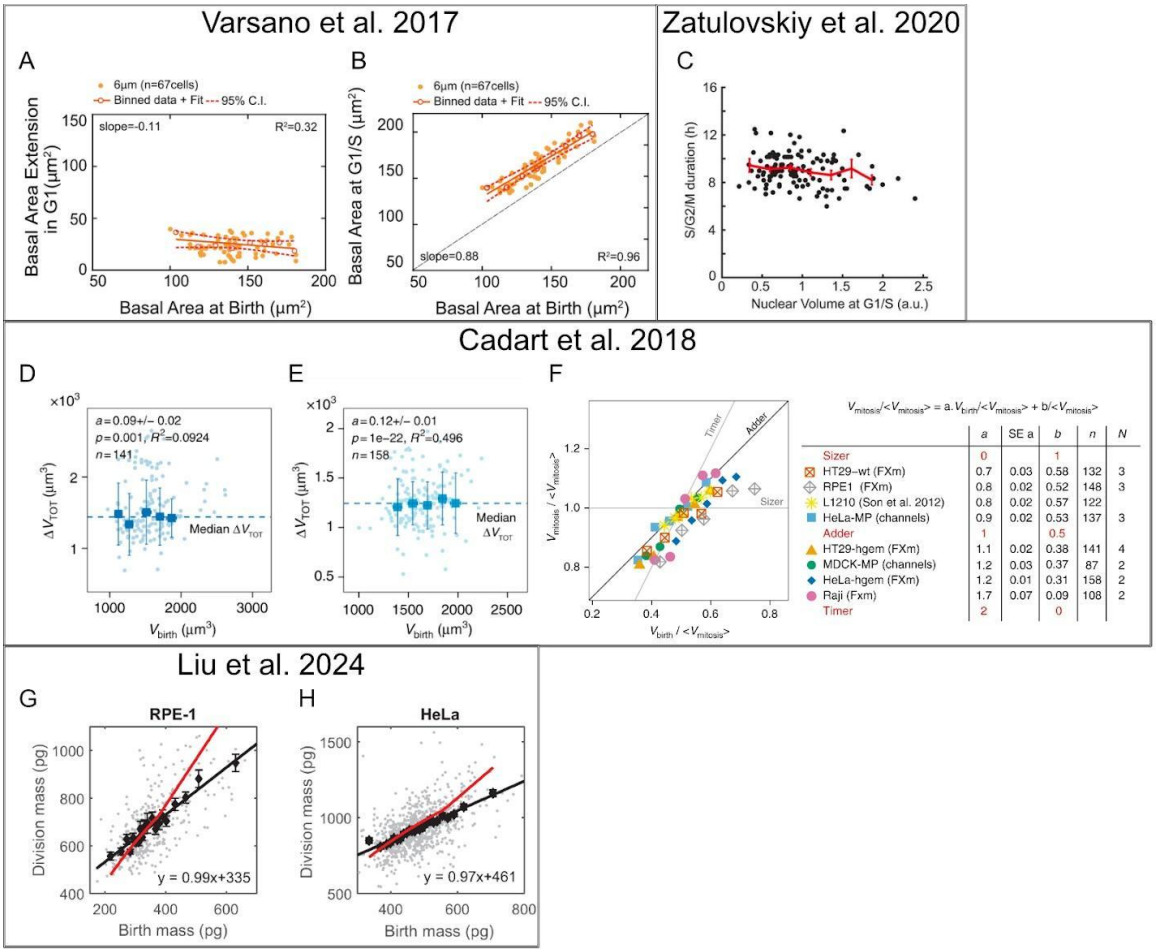

Figure 3: Measurements of single cells showing adder behavior. A,B) Varsano et al. 2017, figure 5 a,b, Rat Basophilic Leukemia cells (RBL) Basal area at birth vs. Basal area extended during G1 (A) and Basal area at birth vs. Basal area at G1/S (B). C) Zatulovskiy et al. 2020, figure 3E, nuclear volume at G1/S transition vs S/G2/M duration. D,E,F) Cadart et al. 2018, figure 5g,h volume at birth vs total change in volume through cell cycle for HT29-gem (F) and HeLa-gem (E). figure 2b, Left graph: plot of volume at mitosis vs. volume at birth rescaled by the mean volume at mitosis for various cultured mammalian cell lines. Ideal slopes for stereotypical homeostatic behaviors are shown as black and gray lines (F). G,H) Liu et al. 2024 figure 3I,J, birth mass vs division mass (pg) for RPE-1 (G) and HeLa (H).

If growth control occurs throughout the cell cycle, under the adder model, size at birth will be proportional to size at mitosis. Cadart et al. used FXm to show a direct correlation between size at birth and size at mitosis for both cell lines (1)(Figure 3F) and primary cells (1). Liu et al. have shown a direct correlation between birth mass and division mass by ceQPM (2)(Figure 3G,H). They also found a decrease in variability of cell size, measured by CV, throughout the cell cycle, suggesting size control is present outside of G1

phase (2). Cell size at birth correlating with size at mitosis suggests that the amount of growth occurring during the cell cycle is independent of starting size, in direct support of the adder model.

Another feature of the adder model is an amount of total growth that is independent of starting cell size. Varsano et al. identified that total growth in G1 was constant, regardless of size at birth (3)(Figure 3A). Although Cadart and colleagues observed a slightly negative correlation between change in volume during G1 versus volume at birth, they observed a positive correlation between change in volume during S/G2 and volume at the G1/S transition. Similarly, when they compared volume at birth to volume at G1/S transition, the proportionality between the two fit closer to an adder model in cell lines and fit perfectly for primary cells (1). In concordance with the adder model, they noticed a constant amount of growth independent of cell size at birth, in both HT-29 and HeLa cell lines (1)(Figure 3D,E).

Direct measurement of single cells has shown that: 1) Cell size at the G1/S transition is directly proportional to birth size, 2) Birth size is directly proportional to size at mitosis, 3) Size accumulation throughout the cell cycle is constant and independent of size at birth. Taken together, these data show strong support for adder-based size control at a single-cell level, through orthogonal assays. Although there are single-cell measurements through orthogonal methods that suggest an adder model could explain size control in mammalian cells, no biochemical evidence exists giving a mechanistic insight to how this control is achieved. Efforts need to be made to test a biochemical

mechanism to better understand adder-like control in mammalian cells, as has been done in *S. cerevisiae* (9,10).

Summary:

Recent work has shined a new light on the regulation of size control in mammalian cells, yet it remains to be seen how cells maintain a homeostatic size. Both sizer and adder models are yet to be proven, or disproven.

Little evidence at a single cell level exists of sizer control and only exists under specific, chemically-induced conditions or in vivo with low resolution. These data show growth accumulated during different phases to be proportional to the size at the start of these phases, arguing against what has been seen by other groups, yet wide distributions of sizes have been seen at each checkpoint. Suggesting cells might modulate growth based on size, however, as a specific size threshold has not been observed, commitment to progression through the cell cycle is not dependent on a particular size. A dilution model has been suggested as a biochemical mechanism linking size to cell cycle progression. Such a mechanism seems tenuous as dilution could be corollary and not causative of cell cycle entry, and may not be potent enough of a signal to trigger such a significant event as commitment to divide. In addition, it is unclear how a small increase in cytoplasmic volume could dilute a transcriptional inhibitor that is tightly bound to chromatin. There is no biochemical evidence or models to explain how an increase in cytoplasmic volume could influence the activity of a transcription

factor bound to chromatin. Without this kind of mechanistic data, the dilution model remains hypothetical.

As more evidence emerges, it is increasingly apparent that the adder model more closely describes mammalian cell size control. Direct single-cell evidence using orthogonal methods have shown adder-like size control across various cell lines and primary cells through these methods, although no mechanism has been suggested. Size at G1/S and size at mitosis are directly proportional to size at birth, and total amount of growth throughout the cell cycle is constant and independent of starting size. An important path for the future of the adder model would be interrogation to a potential mechanism of how cells measure growth accumulated, which has been seen in yeast (9,10). Elucidating a mechanism in mammalian cells would greatly increase the likelihood this model describes size control, and is feasible as cell size control is a fundamental aspect of a cells basic biology.

To further distinguish between the models proposed, a comprehensive analysis of various cell types over multiple generations, at a single-cell level needs to be done. An example of this would be centrifugal elutriation to separate cells based on size, then tracking single-cell changes in size throughout the cell cycle for multiple generations, for various cell-lines and primary cells.

Citations:

1. Cadart, C., Monnier, S., Grilli, J., Sáez, P. J., Srivastava, N., Attia, R., Terriac, E., Baum, B., Cosentino-Lagomarsino, M., & Piel, M. (2018). Size control in mammalian cells involves modulation of both growth rate and cell cycle duration. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-05393-0

2. Liu X, Yan J, Kirschner MW (2024) Cell size homeostasis is tightly controlled throughout the cell cycle. PLoS Biol 22(1): e3002453. https://doi.org/10.1371/journal.pbio.3002453

3. Varsano, G., Wang, Y., & Wu, M. (2017). Probing Mammalian Cell Size Homeostasis by Channel-Assisted Cell Reshaping. *Cell Reports*, *20*(2), 397–410. https://doi.org/10.1016/j.celrep.2017.06.057

4. Zatulovskiy, E., Zhang, S., Berenson, D. F., Topacio, B. R., & Skotheim, J. M. (2020). Cell growth dilutes the cell cycle inhibitor Rb to trigger cell division. *Science*, *369*(6502), 466–471. https://doi.org/10.1126/science.aaz6213

5. Tan, C., Ginzberg, M. B., Webster, R., Iyengar, S., Liu, S., Papadopoli, D., Concannon, J., Wang, Y., Auld, D. S., Jenkins, J. L., Rost, H., Topisirovic, I., Hilfinger, A., Derry, W. B., Patel, N., & Kafri, R. (2021). Cell size homeostasis is maintained by CDK4-dependent activation of p38 MAPK. *Developmental Cell*, *56*(12), 1756-1769.e7. https://doi.org/10.1016/j.devcel.2021.04.030

6. Xie, S., & Skotheim, J. M. (2020). A G1 Sizer Coordinates Growth and Division in the Mouse Epidermis. *Current Biology*, *30*(5), 916-924.e2. https://doi.org/10.1016/j.cub.2019.12.062

7. Dolznig, H., Grebien, F., Sauer, T., Beug, H., & Müllner, E. W. (2004). Evidence for a size-sensing mechanism in animal cells. *Nature Cell Biology*, *6*(9), 899–905. https://doi.org/10.1038/ncb1166

8. Schaechter, M., & Koch, A. L. (1962). A model for statistics of the cell division process. Journal of General Microbiology, 29, 435–454. http://www.ncbi.nlm.nih.gov/pubmed/14033999

9. Anastasia, S. D., Nguyen, D. L., Thai, V., Meloy, M., MacDonough, T., & Kellogg, D. R. (2012). A link between mitotic entry and membrane growth suggests a novel model for cell size control. *Journal of Cell Biology*, *197*(1), 89–104. https://doi.org/10.1083/jcb.201108108

10. Jasani, A., Huynh, T., & Kellogg, D. R. (2020). Growth-Dependent Activation of Protein Kinases Suggests a Mechanism for Measuring Cell Growth. *Genetics*, *215*(3), 729–746. https://doi.org/10.1534/genetics.120.303200