Title: Cell size sensing in animal cells coordinates anabolic growth rates with cell cycle progression to maintain uniformity of cell size.

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Abstract

The uniformity of cell size in healthy tissues suggests that control mechanisms might coordinate

2   cell growth and division. We derived a method to assay whether growth rates of individual cells

depend on cell size, by combining time-lapse microscopy and immunofluorescence to monitor

4   how variance in cell size changes as cells grow. This analysis revealed two periods in the cell

cycle when cell size variance decreases in a manner incompatible with unregulated growth,

6   suggesting that cells sense their own size and adjust their growth rate to correct aberrations.

Monitoring nuclear growth in live cells confirmed that these decreases in variance reflect a

8   process that selectively inhibits the growth of large cells while accelerating growth of small cells.

We also detected cell-size-dependent adjustments of G1 length, which further reduce variability.

10   Combining our assays with chemical and genetic perturbations confirmed that cells employ two

strategies, adjusting both cell cycle length and growth rate, to maintain the appropriate size.

12   Introduction:

Uniformity of cell size is a consistent feature of healthy tissues. While different cell types

14   can differ greatly in size, cells within a given tissue tend to be strikingly similar (1). In some

tissues, loss of cell size uniformity is a characteristic of malignancy(2). Such observations raise

the intriguing question of whether there are dedicated mechanisms that restrict cell size to a

2 specific range. Is size uniformity the product of cellular processes that monitor cell size and

correct deviations from a target size(1,3)?

4 Studies of yeast have long postulated the existence of a cell size checkpoint in the *S. cerevisiae*(4,5). In contrast, studies of animal cell size have remained inconclusive as to whether

6 animal cells employ any form of size sensing (1,3). As early as 1965, A. Zetterberg published

evidence consistent with the existence of a cell size checkpoint in human fibroblasts(6,7). Later

8 literature, however, has predominantly suggested the opposite view, i.e. that growth and cell

cycle progression are not coordinated(8–10). Similarly, in 2013 our group developed a

10 mathematical analysis (ergodic rate analysis, ERA) that predicted the existence of a size-

discriminatory process that coincides with S phase entry(11). However, since predictions of ERA

12 are contingent on several assumptions, these findings did not resolve the controversy

surrounding this topic(1,3). Equally important, while ERA hinted at the existence of a process

14 that is influenced by cell size, it provided little insight into the nature of that process. This

ambiguity was reflected in a more recent review in *Cell* concluding that "There is little evidence

16 that a cell directly senses or uses some type of ruler to measure its size"(3). While these long-

lasting controversies reflect the interest in the subject of size sensing, they also highlight the

18 conceptual challenge impeding this field of study: what are the experimental criteria for

detecting cell size sensing?

20 While it is easy to envision experimental assays of cell size, assays of size *sensing* are

more challenging to conceptualize. In this study, we describe experimental approaches to assay

22 size sensing by monitoring cell size variance. We also develop methods to separately assay the

influence of cell size on cell cycle progression and on growth rate. With these approaches, we

determine that animal cells monitor their size and correct deviations in cell size. Our results show

2   that, like S. cerevisiae, animal cells that are smaller than their target size spend longer periods of

growth in G1. Surprisingly, however, we found that in addition to a G1 length extension in small

4   cells, animal cells also employ a conceptually different strategy of size correction. During two

distinct points in cell cycle, anabolic growth rates are transiently adjusted so that small cells

6   grow faster and large cells grow slower. These periods of growth rate adjustments function to

lower cell size variability, promoting size uniformity. To our knowledge, such a reciprocal

8   coordination of growth rate with cell size has not been previously observed in any organism.

10   Results:

A size threshold regulates S-phase entry in animal cells.

12   Previous literature has defined cell size as cell volume(12,13) or cell mass(14). In this

study, we define cell size by a cell's total macromolecular protein mass, as this metric most

14   closely reflects the sum of anabolic processes typically associated with cell growth(15) and with

activity in growth promoting pathways such as mTOR(16). In contrast, cell volume is a more

16   labile phenotype, sensitive to ion channel regulation and fluctuations in extracellular osmolarity.

        If cell size checkpoints do not exist, and the size of a cell is not a determinant of S phase

18   entry, the reason that S phase cells are larger than G1 cells must be that S-phase cells have had

more time to grow since their last division. Thus, to test whether a cell size checkpoint regulates

20   S phase entry we compared cells in S-phase to cells of the same age (i.e. time elapsed since last

division) that are still in G1.

To measure cell size together with the amount of time elapsed since a cell's last division, we took the following approach. We used time-lapse microscopy to image live HeLa and Rpe1 cells for 1-3 days, and then immediately fixed them and applied AlexaFluor 647-Succinimidyl Ester (SE-A647), a quantitative protein stain that we previously established as a an accurate measure of cell mass (11). (For more supporting evidence on the use of this method, see supplementary file 1-1.) We computationally tracked thousands of individual cells over the course of the time-lapse movies and recorded the amount of time that elapsed between each cell's "birth" via mitosis and its fixation, which we will refer to as the cell's "age". To distinguish S-phase cells from cells in G1, we used cell lines stably expressing mAG-hGem(17), a fluorescent reporter of APC activation and G1 exit. This experimental design allowed us to measure the age, size, and cell cycle stage of each cell at the time of fixation.

Figure 1A shows the average cell size as a function cell age (i.e. time since division). As expected, the average size of the oldest cells is double that of the youngest. The transient slowing of cell growth about 10 hours after birth (the average age of S-phase entry) is consistent with a behavior we and others(11,18) previously identified. In figure 1B, cell size vs. age (time since division) is plotted separately for G1 and post-G1 cells. As expected, cells in the first hours post-"birth" appear exclusively in the G1 stage, while later time points are characterized by heterogeneity in cell cycle stage, with some cells still in G1 while others have transitioned into S phase. Consistent with the existence of a cell size checkpoint, Fig 1B shows that S phase cells are significantly larger than G1 cells of the same age, even though both have been growing for the same amount of time. This result suggests that cells exit G1 in a size-dependent manner. Furthermore, the mean size of G1 cells plateaus as cells begins to enter S-phase, consistent with a mechanism where cells enter S-phase upon reaching a particular size threshold. The size

difference between identically aged G1 and S phase cells is statistically significant (student's t-

2   test p< 3.92e-04) and was observed in three additional independent replicate experiments

(p<0.009, p<5.80e-08, p<9.07e-15).

4   If cells enter S-phase only after attaining a threshold size, cells that are born small are

expected to have longer G1 periods. To test this, we assayed the correlation of cell size at birth

6   with a direct measurement of G1-length in single cells. Since we cannot use the protein staining

technique in live cells, we measured the size of the nucleus as a proxy for cell size, using time-

8   lapse microscopy. In yeast, the nucleus is known to grow continuously throughout all stages of

cell cycle and is correlated with cell size(19), and we found that the same is true in our

10   experimental model (supplementary file 1-2). To further verify that nuclear growth accompanies

cellular growth, independent of cell cycle progression, we arrested the cell cycle with aphidicolin

12   and still observed a dramatic increase in nuclear size as cells grew (supplementary file 1-fig.

S2C). When we monitored nuclear growth in single cells expressing nuclear-localized cell cycle

14   markers(17), we observed a negative correlation between size of the nucleus at birth and G1

duration (fig. 1C). This result confirms that cells that are smaller at birth compensate with

16   increased periods of growth in G1 and is consistent with the model of a cell size threshold at G1

exit.

18   To further test whether information about cell size is communicated to the cell cycle

machinery, we examined the effect of slowing down cellular growth rates on the length of G1. If

20   cells leave G1 only when a particular size has been reached, slowing down their growth rate is

expected to prolong G1, as cells would require more time to reach the threshold size. Growth rate

22   can be slowed down with drugs such as the mTORC1 inhibitor rapamycin. Culturing cells in 70

nM rapamycin caused a 60% decrease in the average growth rate and a compensatory 80%

2   increase in G1 duration, resulting in only a 20% reduction in cell size (fig. 2A-E).

        Figure 2 shows that the influence of rapamycin on growth rate and on G1 length is far

4   more dramatic than its influence on cell size. Previous studies have already shown that in

addition to its effect on cell size, rapamycin also alters G1 length(20–22). In those studies, the

6   dual influence of rapamycin on both size and G1 length were interpreted to suggest two

independent (pleiotropic) functions of the drug(23–25). However, our current results suggest an

8   alternative, more parsimonious interpretation. Namely, that the influence of rapamycin on G1

length is an indirect consequence of its influence on growth rate. According to this interpretation,

10   the inhibition of cell growth by rapamycin results in a gradual reduction of cell size which, in

turn, triggers compensatory increases in G1. This new interpretation is consistent with the

12   negative correlation that we observe between cell size and G1 length, even in the absence of

rapamycin (fig. 1B,C). Additional support for this new interpretation will be provided in a later

14   section of this study (fig. 5C,F), where we show that while the influence of rapamycin on growth

rate is observed immediately after exposure of cells to the drug, the influence of rapamycin on

16   G1 length is observed only after a significant delay. This suggests that G1 length extension is

only indirectly related to the rapamycin treatment, and is mediated by some property that

18   changes slowly over time, presumably cell size. Also in figure 5, we will show that this

relationship of growth rate and cell size is persistent across a larger variety of pharmacological

20   and genetic perturbations.

The rate of cell growth is adjusted in a size-dependent manner

22   The results described above suggest that size uniformity of animal cells is maintained by

keeping small cells in G1 for longer periods of growth. However, terminally differentiated

animal cells do not divide and yet, often undergo precise adjustments of cell size(26). This

2   suggests the presence of size specification mechanisms that are not dependent on regular cell

divisions. Furthermore, the size threshold model is fundamentally limited in its ability to correct

4   the size of very large cells. If some cells grow fast enough to double even in a very short cell

cycle, regulation of cell cycle length alone cannot constrain size variability in the population.

6   The size of a cell is the product of both growth duration (cell cycle length) and growth

rate. If cells have a mechanism to sense their own size, it may be that not only G1 length but also

8   cellular growth rate is adjusted accordingly, to achieve the appropriate cell size. To investigate

this possibility, we examined the relationship between cell size and growth rate.

10   If a cell can sense its own size and adjust its growth rate accordingly – just as a

thermostat coordinates heat production with room temperature via short bursts of heat– we might

12   expect these corrections to be transient and subtle. Experimental detection of transient changes in

growth rates of single cells is difficult. To circumvent this difficulty, we derived an indirect

14   inference method to assay whether growth rates of individual cells are dependent on cell size.

Using the coupled measurements of cell size and cell age described above (fig. 1), we sorted

16   cells based on their ages (i.e. time since division) and calculated the variance in cell size as a

function of age.

18   In the absence of any size-dependent growth rate regulation, the variance in cell size is

expected to increase with age, as cells grow, since individual cells in a population will vary in

20   their growth rates. In any given time interval, fast-growing cells will accumulate more mass than

slow-growing cells, thereby increasing disparities in cell size (fig. 3A). In fact, variance in size

22   can only decrease if small cells grow faster than large cells (fig. 3B). This can be demonstrated

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by considering a time interval during which cells grow from S_1 to S_2. Cell size variance at any

2   given time t_2 is related to the cell size variance at any previous time t_1 by:

4   Var (S_2) = Var (S_1) + Var (ΔS) + 2Cov $(S_1, \Delta S)$ (Eq. 1.0)

6   Therefore, the change in size variance follows:

8   $\Delta Var = Var(S_2) - Var(S_1)$

$\qquad = Var(\Delta S) + 2Cov(S_1, \Delta S)$ (Eq. 1.1)

10

where ΔS is the change in cell size accruing over the time period Δt, i.e. the growth rate. Since

12   the variance in growth rates, Var (ΔS), is always positive (by definition), cell size variance can

decrease with time (ΔVar <0) if (and only if) the correlation of cell size and growth rate is

14   negative, i.e. Cov$(S_1, \Delta S) < 0$. This mathematical relationship can be exploited to experimentally

detect periods of size-dependent growth rate regulation without directly measuring growth rates.

16   Figure 3 shows that periods of decreasing cell size variance do, in fact, occur during the

cell cycle of both HeLa and RPE1 (Fig. 3C-F). During these periods, cells grow (mean size

18   increases), and yet become more similar in size. This indicates that cellular growth rates are

regulated to correct aberrant cell sizes. To quantify the strength of this regulation, we normalized

20   the change in size variance, $\Delta Var = Var(S_2) - Var(S_1)$, by the amount of growth that has

occurred (i.e. the change in mean size, $\bar{S}$) to define the *coefficient of growth rate variation*,

22   designated G_{cv}.

8

$$G_{cv} = \begin{cases} \dfrac{\sqrt{\Delta Var}}{\bar{S}_2 - \bar{S}_1} & \Delta Var > 0 \\[2em] -\dfrac{\sqrt{|\Delta Var|}}{\bar{S}_2 - \bar{S}_1} & \Delta Var < 0 \end{cases}$$

(Eq. 2)

2

The G_{cv}-value analysis is a new method to interrogate size control in growing cells. As

4 long as the mean cell size is increasing over time, G_{cv} can be interpreted as follows. If a cell's

growth rate is independent of its size, G_{cv} directly equals the coefficient of variation (CV) of

6 cellular growth rates. This can be shown by substituting the relationship in eq. 1 into the formula

for G_{cv}, noting that, if growth rates are size-independent, variance will increase over time and

8 $\text{Cov}(S_1, \Delta S) = 0$. In this case, $G_{cv} = \dfrac{\sqrt{Var(\Delta S)}}{\bar{S}_2 - \bar{S}_1}$.

Negative values of G_{cv} (arising from dips in variance) imply that growth rate is actually

10 not independent of size, and that cell size and subsequent growth rate are negatively correlated.

We can also note that if G_{cv} is much higher than is plausible for the CV of growth rates (which

12 will equal 1 if growth is a Poisson process), it is likely that growth is positively correlated with

size, as in the case of exponential growth.

14 Plotting G_{cv} versus cell age consistently reveals two distinct periods during which cell

size and growth rate are negatively correlated (i.e. G_{cv} is negative), in both HeLa and Rpe1 cells

16 (Fig. 3G,H). This result is striking, because it suggests communication between cell size and

cellular growth rate that is transiently established twice during the cell cycle, perhaps due to cell-

18 cycle-dependent signalling linking size and growth rates.

The analysis presented in figure 3 does not discriminate G1 cells from S phase cells and,

20 consequently, the observed drops in variance cannot be explained by a cell-size checkpoint

gating G1. Therefore, figure 3 clearly reveals two distinct times in the cell cycle, where the

growth rate of individual cells is selectively repressed in large cells or accelerated in small cells –

2   increasing uniformity of cell size. The only alternative possibility is that cells are removed from

the distribution, by either dividing or dying in a size-dependent manner. This is very unlikely to

4   be the case, since the dips occur earlier than cells start dividing. (This is true for at least the first

dip in Rpe1 and both dips in HeLa, where the mean cell cycle length is 21 hrs, with a standard

6   deviation of 1.6 hrs. See supplementary file 1-3 for a sample cell cycle length distribution.)

There was also a very low rate of apoptosis (<1% of cells imaged from birth that died before

8   dividing).

       The results of the G_{cv}-value analysis are surprising. G1 checkpoints that link cell cycle

10   progression to cell size have been previously observed in yeast(4). A few reports have suggested

that G1 duration depends on growth rate rather than cell size(27,28). However, a process that

12   stabilizes cell size by transient corrections of growth rate has not been previously observed in

any organism. In fact, such a possibility was never previously hypothesized except in our own

14   previous publication of ERA(11), but since ERA is fundamentally incapable of distinguishing

changes in growth rate from changes in cell cycle progression rate, that suggestion remained a

16   speculation. As such, this result represents the first clear evidence that cellular growth rates are

adjusted in a cell-size-dependent manner to increase uniformity in a population.

18   To test the conclusions of the G_{cv}-value analysis, we asked whether the correlation of

growth rate and cell size could be observed directly in live cells. Using time-lapse microscopy,

20   we monitored nuclear growth in hundreds of live cells over several days. Comparing growth

trajectories collected from the largest and smallest cells in the population provided a dramatic

22   demonstration that growth rates are, indeed, reciprocally coordinated with cell size (fig. 3I).

Because growth rates became slower in large cells and faster in small cells, individual cells that

10

were initially quite different in size gradually become more uniform in size, explaining the

2   decreases in cell size variance shown in figure 3E-H. Furthermore, as predicted by the G_{cv}-value

analysis, the negative correlation of cell size and subsequent growth rate was not continuously

4   present, but was transiently established twice during the cell cycle (fig. 3J).

Growth rate and cell cycle length are coordinated to maintain cell size at fixed values.

6   The G_{cv}-value analysis and live-cell tracking of nucleus size all indicate that cellular

growth rates are regulated in a manner that reduces cell size variability. Taken together, our

8   results suggest that cells employ two separate strategies to correct deviations from their

appropriate size: (1) small cells spend more time in G1 and (2) small cells grow faster than large

10   cells (fig. 4). A prediction of this dual-mechanism model is that perturbations that slow the cell

cycle would be counteracted by a compensatory decrease in growth rate, allowing cells to

12   accumulate the same amount of mass despite the longer periods of growth. Conversely,

perturbations that reduce growth rate would be counteracted by a compensatory lengthening of

14   G1. To experimentally lengthen the cell cycle, we treated cells with a variety of pharmacological

inhibitors of the cyclin dependent kinases or cdc25. Drugs were used at concentrations that slow

16   the proliferation rate but do not cause cell cycle arrest (supplementary file 1-4). To

experimentally decrease growth rates, we used cycloheximide, as well as the mTOR inhibitors

18   Torin-2 and rapamycin, also at doses where cells maintain viability and proliferation

(supplementary file 1-4). We then monitored growth and proliferation over time, by fixing

20   samples at intervals and measuring the bulk protein content (total SE-A647 intensity of sample)

and number of cells present. From these measurements, we calculated the average growth rate

22   and cell cycle length of cells in each condition. Cell cycle length was also independently

measured, by monitoring the proliferation of live cells in each condition with differential phase-

2   contrast microscopy over the course of three days.

Figure 5A,B shows that chemical perturbations of growth rate and perturbations of cell

4   cycle length had surprisingly small effects on cell size. Consistent with the dual-mechanism

model, treatment with all but one of the tested compounds resulted in coordinated changes of

6   both cell cycle length and growth rate, such that normal cell size was maintained (fig. 5C, fig.

6C,D,F,G). Furthermore, while the inhibitors of cell cycle regulators produced an immediate

8   effect on cell cycle length, their effect on growth rate was observed only after prolonged

treatment (Fig. 5E). This suggests that the effect of these inhibitors on growth rate is indirect and

10 is mediated by a property that accumulates over time, presumably cell size. Similarly,

cycloheximide, rapamycin and Torin-2 induced an immediate decrease in growth rate, while the

12 effect of these drugs on cell cycle length was observed after a delay (Fig. 5F). Naively, changing

how fast cell grow or changing the amount of time cells grow should result in proportional

14 changes to cell size. These results, however, demonstrate the surprising stability of cell size, in

the face of perturbations to either growth rate or cell cycle progression, and reveal a

16 compensatory relationship between these two processes.

To further test these conclusions, we overexpressed cyclin E to shorten the duration of

18 cell cycle. Since CDK inhibitors that lengthened cell cycle were counteracted by a reduction in

growth rate, we asked whether shortening the cell cycle would result in an increased growth rate.

20 Figure 5D shows that, as anticipated, the shorter cell cycles caused by cyclin E overexpression

are compensated for by faster rates of cell growth, rendering cell size relatively unchanged.

22 Of the tested perturbations to cell cycle regulators, only the cdk4/6 inhibitor palbociclib

increased cell cycle length without a compensatory decrease in growth rate, causing an unusual

12

increase in cell size (fig. 6A,B). Future studies investigating how palbociclib perturbs the

2 crosstalk between cell cycle progression and cell growth may provide clues to the mechanisms of

cell size sensing.

4   A quantitative prediction of the dual-mechanism model is that growth rate, v, and cell

cycle length, τ, are coordinated to maintain cell size at its fixed target size:

6

$\tau \times v = target\ size.$

8

This means that growth rate and cell size are related by, $v = \frac{C_x}{\tau}$, where C_x is a constant target

10 size. Figure 5 shows that our measurements are in agreement with this prediction. The curves in

figure 5C-F are iso-cell-size contours and represent all paired values of growth rate and cell

12 cycle length that correspond to one particular cell size. A notable feature of figure 5C,D is the

significant influence of drugs on both growth rate and cell cycle length, in contrast to the

14 relatively small influence of those same drugs on cell size. Naively, one would expect a drug that

decreases growth rate by 50% would result in a 50% decrease in cell size. Results from our

16 experiment show that, for many drugs, this expectation is false. While drug treatments produced

up to fourfold changes in both growth rate and cell cycle length, the resulting changes in cell size

18 were mostly under 30%. As mentioned above, while the influence of CDK inhibitors on cell

cycle progression was immediate, their influence on growth rate was observed only after many

20 hours of drug treatment (Fig. 5E), supporting the conclusion that the decrease in growth rate is a

response to the gradual increase in cell size that occurs during a lengthened cell cycle.

22 Conversely, while the influence of mTOR inhibitors on growth rate was immediate, their

influence on cell cycle progression was observed only after a delay (Fig. 5F). These imply that

cells have evolved regulation that buffers cell size from changes in cell cycle length or growth

2   rate. In this light, it may be interesting to examine why cells have evolved to tolerate changes in

cell cycle length and growth rate but not changes in cell size.

4   Finally, in an attempt to disrupt the processes that maintain cell size uniformity, we

treated cells with the HSP90 inhibitor radicicol. HSP90 is known to suppress phenotypic

6   variability(29) and has also been found to regulate both cell cycle progression(30,31) and

mTORC1 mediated growth in response to cellular stress(32). Remarkably, culturing cells in

8   radicicol not only decoupled growth rate and cell cycle length, but also resulted in a marked

increase in cell size heterogeneity (fig. 6A,E). This finding illustrates that cell size uniformity is

10   a regulated phenotype that can be perturbed by disrupting the coordination of growth and cell

cycle progression.

12

Discussion:

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The subject of cell size uniformity has been largely unexplored. It was not even clear

16   whether size uniformity is important or why size is so uniform despite variation in growth rates

and cell cycle lengths. In this study we show that cells that have escaped their appropriate size

18   range are corrected by two separate mechanisms: regulation of G1 length and regulation of

growth rate. To reveal these mechanisms, we circumvented the experimental difficulty of

20   measuring cellular growth rates by developing methods that infer growth rate regulation from

simpler measurements. With these approaches, we not only provide the first definitive proof that

22   the size of an individual animal cell is autonomously monitored and regulated, but also provide

accessible experimental assays for the study of growth rate regulation.

The stability of cell size in proliferating populations is a clear refutation of the notion that

2   size is simply the quotient of growth and division rates, which are independently regulated with

no feedback between them(10). Not only is there feedback, but it robustly maintains cell size

4   despite strong perturbations of growth and cell cycle progression. The feedback that maintains

size and size variation in growing cells suggests the presence of a size sensor. With an

6   understanding that this sensor feeds back on both growth and cell cycle progression in opposite

ways, and with assays that can separately quantify each mode of regulation, we are in a position

8   to uncover its molecular identity.

In tissues, growth and shrinkage are often due to control of cell proliferation and

10   apoptosis but some cell types such as neurons and muscle cells are controlled by cell growth

rather than proliferation. Hence, in non-dividing cells that maintain size we may find part of the

12   circuitry used in proliferating cells. Although pathways, such as mTOR, that promote cell growth

have been delineated(16,33), it remains unclear how a common set of pathways may specify a

14   different size in each cell type, while ensuring precise uniformity within cells of a common type.

The approaches developed here can be combined with perturbations of these pathways to answer

16   this fundamental question. With that knowledge, we can begin to explore the consequences of

cell non-uniformity in normal cell function and particularly in development, disease, and aging.

18

20   <u>Methods:</u>

22   *Cell culture:* Cells were grown in Dulbecco's Modification of Eagles Medium (Cellgro, 10-017-

CV), supplemented with 10% Fetal Bovine Serum (FBS, Gibco, 26140) and 5%

15

Penicillin/Streptomycin (Cellgro 30-002-CI), incubated at 37°C with 5% CO2. All

2   measurements were made when cells were 50-75% confluent, to avoid the effects of sparse or

dense culture on cell growth and proliferation.

4   *Cell cycle markers*: To distinguish S-phase cells from cells in G1, we used cell lines stably

expressing nuclear-localized fluorescent cell cycle markers (A. Sakaue-Sawano *et al.*, *Cell*. 132

6   (2008) 487–498).

Time-lapse microscopy: Cells were seeded in 6-well, glass-bottom (No. 1.5) plates one day prior

8   to imaging. Leibovitz's L-15 Medium (ThermoFisher, 21083027) with 10% FBS was used

during image acquisition, with a layer of mineral oil on top of the media to prevent evaporation.

10   Microscope was fitted with an incubation chamber warmed to 37°C. Widefield fluorescence and

phase contrast images were collected at 15 minute intervals on a Nikon Ti motorized inverted

12   microscope, with a Nikon Plan Fluor 10x 0.3 NA objective lens and the Perfect Focus System for

maintenance of focus over time. A Lumencor SOLA light engine was used for fluorescence

14   illumination, and a Prior LED light source was used for transmitted light. A Prior Proscan III

motorized stage was used to collect images at multiple positions in the plate during each interval.

16   Images were acquired with a Hamamatsu ORCA-ER cooled-CCD camera controlled by

MetaMorph software. After two days of time-lapse imaging, cells were fixed and stained as

18   described below. Images of fixed cells were acquired on the same microscope, at the same stage

positions, and an image-registration tool was designed in Matlab to match individual cells in the

20   timelapse movies to the corresponding cells in the fixed images.

Fixation and staining: Cells were fixed in 4% paraformaldehyde (Alfa Aesar, 30525-89-4) for 10

22   minutes, then permeabilized in chilled methanol for 5 minutes. Cells were stained with 0.04

ug/mL Alexa Fluor 647 carboxylic acid, succinimidyl ester (SE-A647, Invitrogen A-20006), to

label protein. DNA was stained with DAPI (Sigma D8417).

Pharmacological and genetic perturbations: To slow growth rate the following drug treatments

were used: cycloheximide (1, 0.6, and 0.06 uM, Sigma C4859), Torin 2 (10, 5, and 2.5 nM,

Tocris 4248), rapamycin (7, 0.7, and 0.07 uM, CalBiochem 553211). To slow the cell cycle, the

following drug treatments were used: BN82002 (25, 12.5, 6.2, 3.1, 1.6, and 0.78 uM,

Calbiochem 217691), SNS032 (39 and 9.8 nM, Selleckchem S1145), PHA848125 (175 nM,

Selleckchem S2751), Cdk2 Inhibitor III (5, 1.5, 0.75, and 0.38 uM, Calbiochem 238803),

Dinaciclib (10, 5, and 2.5 nM, Selleckchem S2768), Palbociclib (500, 250, 125, and 62.5 nM,

Selleckchem S1116).

To promote cell cycle progression, cells were transfected with plasmids encoding wild-type or

degradation-resistant cyclin E under control of a constitutive promoter. Cyclin E-pBI-EGFP was

a gift from Bert Vogelstein (Addgene plasmid # 16654)(Rajagopalan, H. *et al. Nature* 428

(2004) 77–81). MIGR1-cyclin E-AA was a gift from Alex Minella (Addgene plasmid # 47498)(

Xu, Y., Swartz, K. L., Siu, K. T., Bhattacharyya, M. & Minella, A. C. *Oncogene* 33 (2014)

3161–3171). Since the transfection process can slow proliferation, the growth rate and cell cycle

length of cells overexpressing cyclin E were compared to those of cells transfected with a control

plasmid, pmCherry-N1 (Clontech, 632523). For comparison with drug-treated cells (fig. 5D),

growth rates and cell cycle lengths of transfected cells were normalized so that cells expressing

pmCherry-N1 matched DMSO-treated cells (controls).

Radicicol (Tocris, 1589) treatments were done at concentrations of 250 and 500 nM.

Cells were imaged using a Perkin Elmer Operetta high content microscope, controlled by

Harmony software, with an incubated chamber kept at 37°C and 5% CO_2 during live-cell

imaging. A Xenon lamp was used for fluorescence illumination, and a 740 nm LED light source

2   was used for transmitted light. To monitor proliferation of live cells, differential phase contrast

images were collected every 6-12 hours, over the course of three days, using a 10x 0.4 NA

4   objective lens. A plot of the number of cells vs. time was fitted to an exponential growth model

to calculate the average cell cycle time.

6   Samples were fixed after 14, 44, and 68 hours in each drug. After fixation and staining with SE-

A647 (protein) and DAPI, fluorescence images were collected with a 20x 0.75 NA objective

8   lens. The bulk protein content (total SE-A647 intensity of sample) and number of cells were

measured in each sample. From these measurements, we calculated the average growth rate and

10   cell cycle length of cells in each condition, by fitting all data points (from two replicates of each

condition) to an exponential growth model. Cell cycle length was also independently measured,

12   by monitoring the proliferation of live cells in each condition with differential phase-contrast

microscopy as described above.

14   *Image analysis and data analysis*: All image analysis (cell segmentation, tracking, measurements

of fluorescence intensity and nuclear size) and data analysis was performed with custom-written

16   tools in Matlab. The mean and variance of cell size as a function of age (figure 3) were

calculated by nonparametric regression, as described by Wasserman (34).

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Figure Legends

2

Figure 1. Small cells spend more time in G1. (A) Mean cell size as a function of age in HeLa

4   cells. Shaded region marks 20% and 80% bootstrapping confidence intervals. (B) Mean size of

G1 (red) and post-G1 (blue) HeLa cells as a function of cell age. Post-G1 cells are larger than G1

6   cells of the same age (student's t-test p< 3.92e-04). Data shown in (A) and (B) is a representative

example of four biological replicates (full experimental repeats), n=5537 cells. (C) Size (area

8   covered in widefield fluorescence image) of HeLa nucleus 1.25 hours after birth vs. G1 duration.

Line shows least-squares linear fit. Pearson's r=-0.41, p=6.7×10^{-8}, by two-tailed Student's t-test.

10   Inset shows distribution of Pearson's r-values generated by randomizing the data, with arrow

marking the r-value of non-randomized data. Data shown in (C) is a representative example of

12   four biological replicates, n=158 cells.

14   **Figure 2. Slow growth rate in mTOR-inhibited cells is counteracted by increase in G1**

duration. The effects of 70 nM rapamycin on the distribution of (A) cell size (protein content),

16   (B) average growth rate, (C) G1-length, and (D) proliferation rate in Rpe1 cells. Solid lines in

(B) show mean size vs. age for control and rapamycin-treated cells, while points represent single

18   cell measurements. (E) Bar plot comparing the influence of rapamycin on growth rate, G1

length, and cell size. Bar heights represent mean, error bars represent standard deviation (n=975

20   cells in control, n=1268 cells in rapamycin). Data shown is a representative example of two

biological replicates (full experimental repeats).

22

Figure 3. Cells adjust their growth rates in a size-dependent manner. (A) Variation in

24   growth rates drives an increase in cell size variance over time. (B) Regulation that limits cell size

21

variance must function via cross-talk between growth and cell size in individual cells. (C-H)

2 Mean size, variance, and G_{cv} plotted as a function of age for Rpe1 (C,E,G) and HeLa (D,F,H)

cells. Shaded regions mark 20% and 80% bootstrapping confidence intervals. For Rpe1 plots

4 (C,E,G), n=975 cells, and data shown is a representative example of four biological replicates.

For HeLa plots (D,F,H), n=721 cells, and data shown is a representative example of four

6 biological replicates. (I) Comparison of growth trajectories of smallest and largest cells. HeLa

cells were sorted based on their size at 10 hours after birth. Nuclear size trajectories for the ten

8 largest (red) and smallest (blue) cells are shown. (J) Correlation (Pearson's r) between size and

subsequent growth rate (2.5 hours later), calculated as a function of age (time since birth). Data

10 shown in (I) and (J) is a representative example of four biological replicates, n=158 cells.

12 **Figure 4. Dual-mechanism model of cell size specification.** Data presented here is consistent

with a model where cells sense their own size and employ two strategies, adjusting both growth

14 rate and cell cycle length, to correct aberrant sizes.

16 **Figure 5. Compensatory relationship between cell cycle length and growth rate.** (A-D) Cells

were treated with varying doses of drugs inhibiting cell cycle progression (circles) or protein

18 production (squares). (A) The fold change in cell size (after 44 hrs in drug) vs. fold change in

growth rate, relative to untreated cells, is plotted for each condition. Dotted line denotes

20 proportional relationship expected if there is no coordination between growth and cell cycle

progression. (B) The fold change in cell size (after 44 hrs in drug) vs. fold change in cell cycle

22 length, relative to untreated cells, is plotted for each condition. (C) Mean growth rate vs. mean

cell cycle length are plotted for cells in each condition, calculated from measured increases in

24 bulk protein and number of cells over the course of a 68-hour incubation. Black lines mark iso-

22

cell-size contours. Region between lines spans a 25% shift in cell size. (D) Cells were transfected

2   with plasmids encoding wild-type or degradation-resistant cyclin E under control of a

constitutive promoter (diamonds). Mean growth rate vs. mean cell cycle length are plotted for

4   cells in each condition, overlaid on the plot shown in (C). Calculation of the Pearson correlation

coefficient between log(growth rate) and log(cell cycle length), using the data shown in (D),

6   yields $r = -0.76$, $p = 2.1 \times 10^{-7}$, by two-tailed Student's t-test, indicating a significant inverse

correlation between growth rate and cell cycle length. (E,F) Mean growth rate vs. mean cell

8   cycle length for cells treated with drugs inhibiting cell cycle progression (E) or protein

production (F), calculated for three time intervals during drug treatment: 0-14 hrs, 14-44 hrs, 44-

10   68 hrs. Lines connect time-points in sequential order, with arrowheads pointing to latest time-

point. Data is shown for 25 uM BN82002 (E, green), 1.5 uM Cdk2 Inhibitor III (E, red), 0.06 uM

12   Cycloheximide (F, blue), 10 nM Torin (F, orange), and 7 uM Rapamycin (F, purple). For (A-F),

each treatment was done in duplicate, with several thousand cells in each sample.

14

Figure 6. Perturbing the relationship between cell cycle length and growth rate. (A) Cells

16   were treated with varying doses of palbociclib or radicicol. Mean growth rate vs. mean cell cycle

length are plotted for cells in each condition, overlaid on the plot shown in figure 5D. (B-G)

18   Representative images of cells treated with 500 nM palbociclib (B), 25 uM BN82002 (C), 250

nM radicicol (E), 5 uM Cdk2 Inhibitor III (F), 175 nM PHA848125 (G), and unperturbed cells
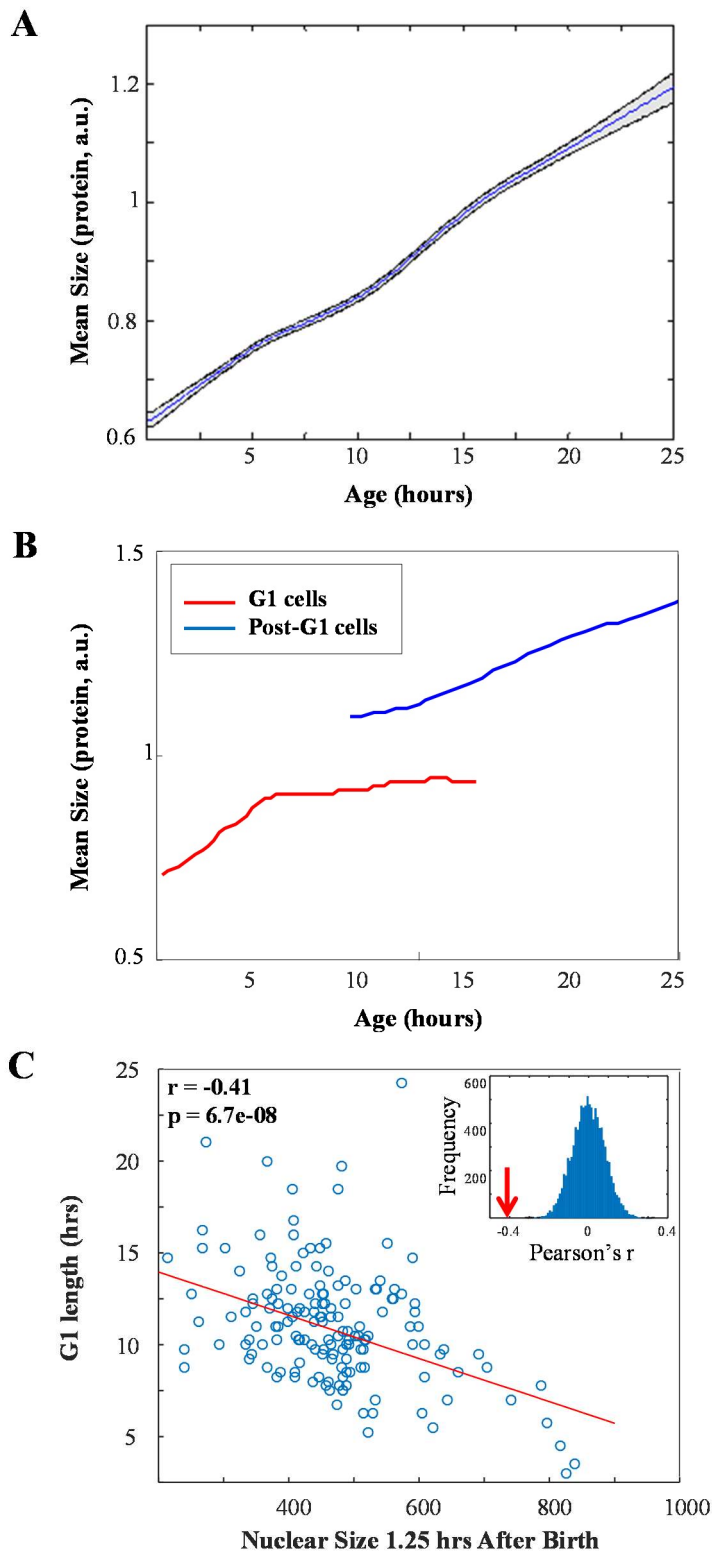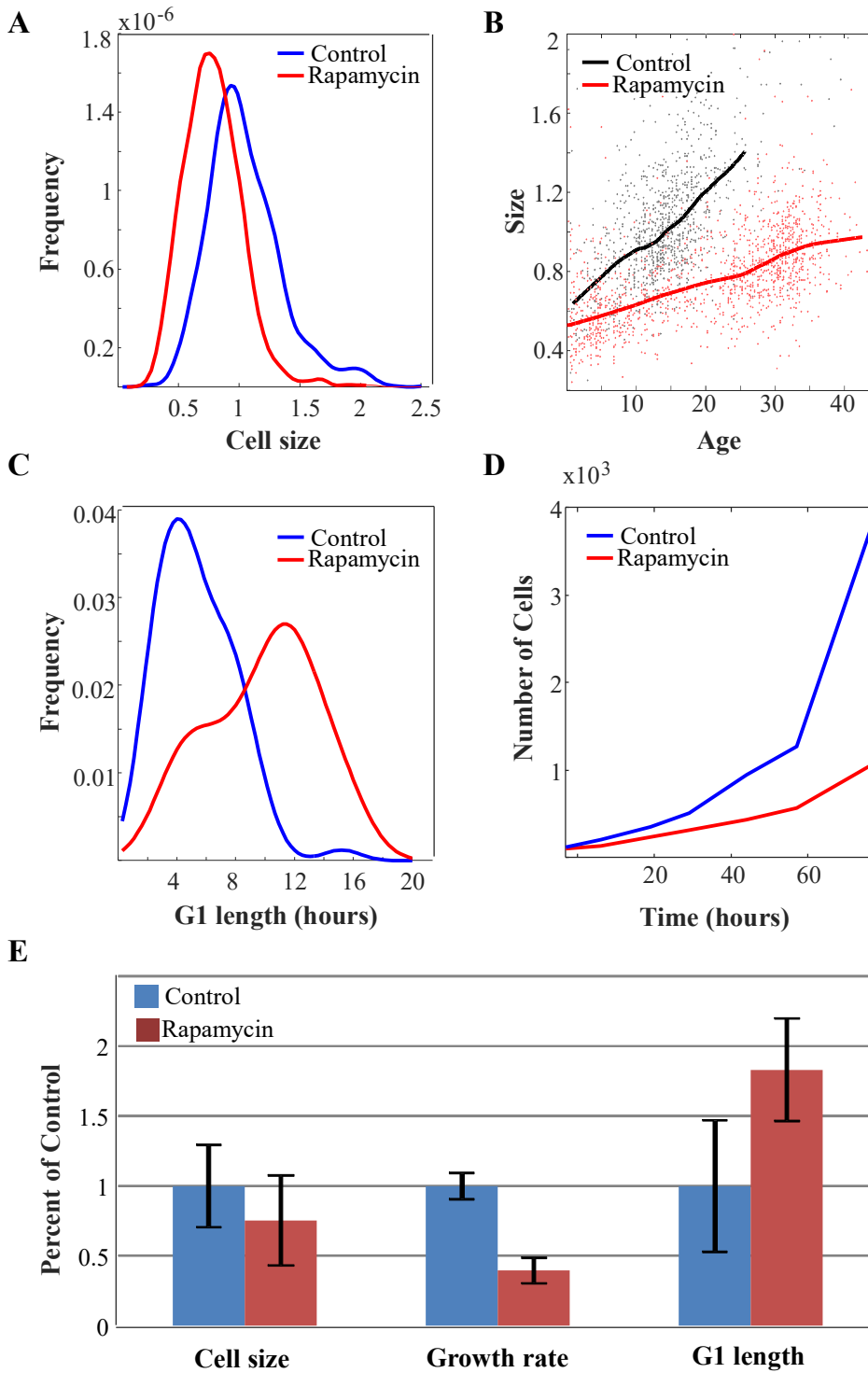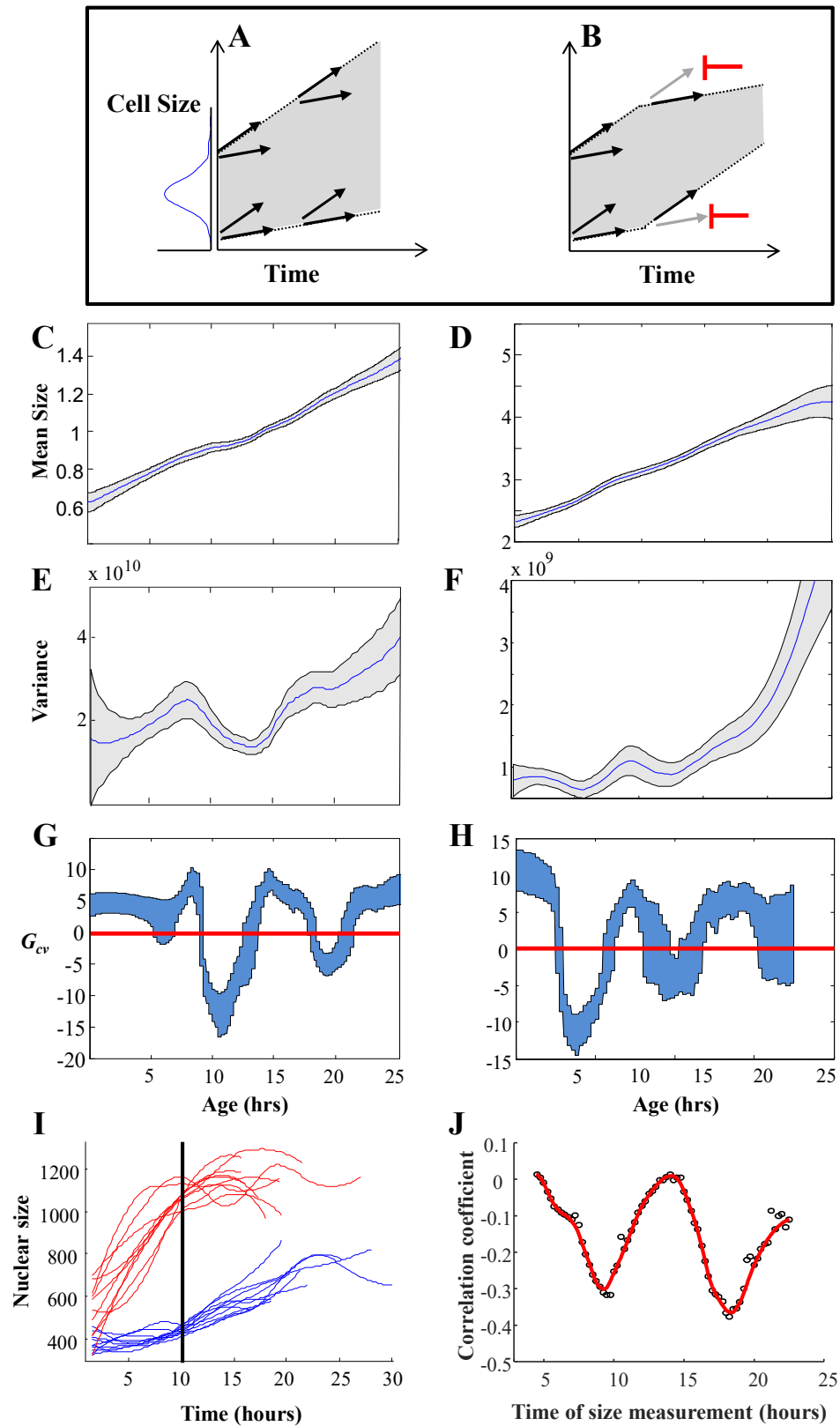
20   (D). Red lines delineate cell boundaries.

Figure 1

A

B

C

Figure 2

Figure 3

Figure 4
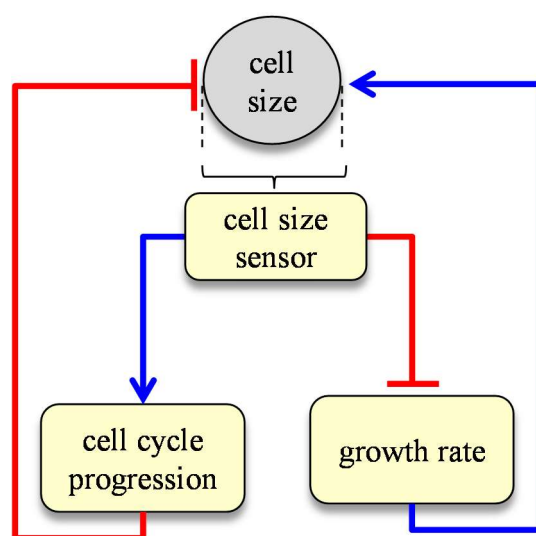
Figure 5
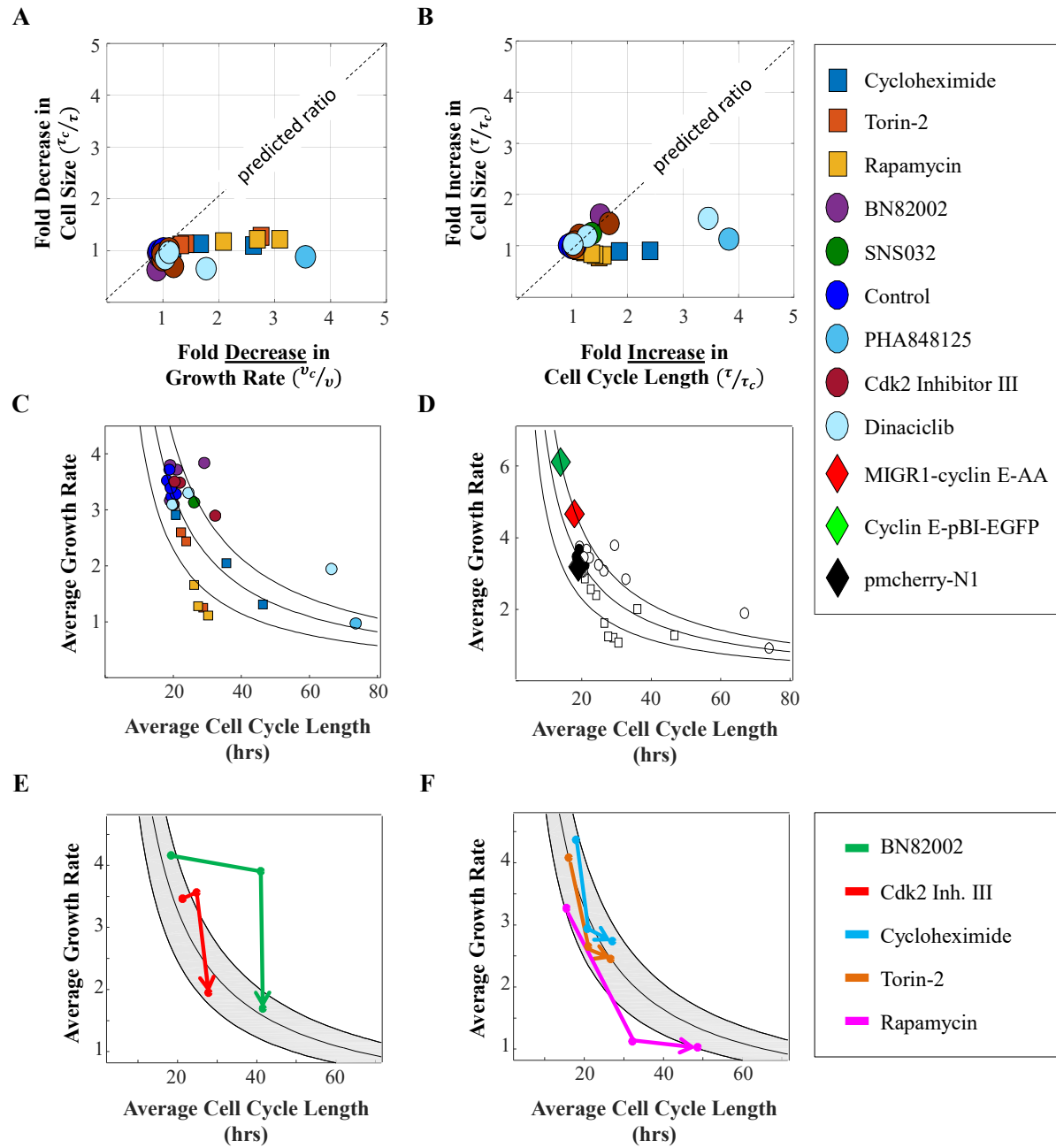
Figure 6

Acknowledgements

Competing Interests

The authors do not have any financial or non-financial competing interests in the publication of this work.