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Genome dilution by cell growth drives starvation-like proteome remodeling in mammalian and yeast cells

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Cell size is tightly controlled in healthy tissues and single-celled organisms, but it remains unclear how cell size influences physiology. Increasing cell size was recently shown to remodel the proteomes of cultured human cells, demonstrating that large and small cells of the same type can be compositionally different. In the present study, we utilize the natural heterogeneity of hepatocyte ploidy and yeast genetics to establish that the ploidy-to-cell size ratio is a highly conserved determinant of proteome composition. In both mammalian and yeast cells, genome dilution by cell growth elicits a starvation-like phenotype, suggesting that growth in large cells is restricted by genome concentration in a manner that mimics a limiting nutrient. Moreover, genome dilution explains some proteomic changes ascribed to yeast aging. Overall, our data indicate that genome concentration drives changes in cell composition independently of external environmental cues.

Cell size is intimately related to cell function and tightly controlled. Macrophages must be large enough to engulf pathogens, whereas lymphocytes must be small enough to squeeze through tight passages. However, apart from such exceptional cases, it is unclear why most cell types adopt their characteristic size. Nevertheless, we know that cell size is tightly controlled in both human tissues[1] and single-celled organisms[2], suggesting its general importance for cell function. For example, budding yeast and mammalian epithelial cells correct for variations in birth size by adjusting cell growth in their G1 phases[3,4]. In fission yeasts, such size regulation takes place in G2 (ref. 5). Each mechanism ensures that cells that were born large or small finish the division cycle at a similar target size.

Despite the ubiquity of size regulation in cell biology, it had been unclear how natural variations in cell size generally affect cell physiology. Apart from the recent discoveries that several cell cycle-regulatory proteins change concentration with cell size[6–12], it was thought that the concentrations of most individual proteins and messenger RNAs were kept constant as cells grow[13–19]. However, recent studies of cultured human cells have potentially revealed a more general link between cell size and cell physiology[20,21]. These studies used mass spectrometry (MS) to show that the concentrations of many, if not most, individual proteins continuously change as cells grow larger, even though total protein concentrations remain roughly constant in the physiological size range[13,18,22–25]. Proteins with concentration increases or decreases with cell size were termed super- and subscaling, respectively (Fig. 1a). Each protein's degree of size scaling can be quantified by regressing its relative change in concentration against the relative change in cell size, producing a slope value (Fig. 1b). A slope value of 1 corresponds to a doubling of the concentration with a doubling cell size, 0 to a constant concentration and −1 to halving the concentration with a doubling of cell size (dilution) (Fig. 1b). The revelation that individual proteins adopt a broad distribution of size-scaling behaviors could help explain

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Fig. 1 | Ploidy-to-cell size ratio, rather than cell size itself, drives conserved changes in mammalian proteome composition. a, Schematic summary of the scaling relationships between protein concentration and cell size as described previously in cultured human cell lines[20]. ER, endoplasmic reticulum. **b**, Derivation of the protein slope value[20]. Protein slopes describe the size-scaling behavior of each individual protein. Proteins with a slope of 0 maintain a constant cellular concentration regardless of cell volume. A slope value of 1 corresponds to an increase in concentration that is proportional to the increase in volume and of −1 to dilution (1/volume). **c**, Schematic illustrating TMT-based proteomic analysis of mouse primary hepatocytes of different size and ploidy. **d**, Small and large hepatocytes with 2*n* or 4*n* genome content isolated using FACS (Extended Data Fig. 1). **e**, PCA of the relative concentration of each protein in small or large G1 cells of either 2*n* or 4*n* ploidy. The PC1 represents gross proteomic change and is plotted against cell volume. Dot size represents the mean cell volume. **f**, Correlation of concentration changes for individual proteins (peptide measurement per protein >5). See Supplementary Table 1 for all 4,684 measured proteins. The $\log_2$(ratio) values represent a protein's concentration change between small and large cells ($n = 1$). Blue dots are *x*-binned data and error bars represent the 99% CI. **g**, Correlation of the size-scaling behaviors of orthologous mammalian proteins (Supplementary Table 2). RPE-1 protein slopes are the mean value of three replicate experiments performed previously by Lanz et al.[20]. The $\log_2$(large:small concentration ratio) is proportional to the protein slope value because the large hepatocytes are approximately twofold larger than the small hepatocytes. Blue dots are manually *x*-binned data and error bars represent the 99% CI. **h**,**i**, Scaling behavior of human (**h**) and mouse (**i**) proteins strictly parsed by subcellular localization. The white dot represents the distribution's median value.

how cell size generally influences cell physiology, namely, large and small cells of the same type are compositionally different.

That the proteome composition continuously changes as cultured human cells grow larger raises three important questions. First, what is the mechanism(s) by which increasing cell size remodels proteome composition? Second, how conserved is this phenomenon? Third, how do cell size-associated compositional changes ultimately reflect changes in cell physiology? In the present study, we demonstrate that changes in genome concentration (that is, ploidy-to-cell size ratio) remodel proteome composition in yeast and mammalian cells. In both yeast and mammalian cells, the dilution of the genome by cell growth elicits a starvation-like phenotype, suggesting that growth in large cells is restricted by genome concentration in a manner analogous to a limiting nutrient. Overall, we conclude that genome concentration is probably a universal determinant of proteome composition in growing cells.

Results

Proteome composition changes with the ploidy-to-size ratio

To test whether size-dependent proteome remodeling is conserved across mammalian cell types, we measured how cell size and ploidy impacted the proteome composition of primary mouse hepatocytes. Mouse hepatocytes were chosen because their ploidy in the mouse liver is heterogeneous, which allows us to simultaneously test the effects of cell size and ploidy in a single experiment. We derived primary cell lines containing both diploid and tetraploid hepatocytes (Fig. 1c). We then measured the proteomes of FACS-isolated, small and large G1 cells that were either diploid or tetraploid (Fig. 1d and Extended Data Fig. 1). Small 4*n* and large 2*n* populations were approximately the same average size, but their proteome compositions were different (Fig. 1e,f and Supplementary Table 1). In contrast, the proteome composition of small 2*n* and small 4*n* cells were similar despite a twofold difference in average size (Fig. 1e,f). Importantly, small 2*n* and small 4*n* cells have a similar ploidy-to-cell size ratio. Consistent with what was previously found in human cell lines, the relative concentration of mouse histone proteins reflected the relative concentration of the genome (Fig. 1f). These results demonstrate that proteome composition changes with the ploidy-to-cell size ratio. This finding is consistent with a previous experiment where human retinal pigment epithelial (RPE)-1 cells were chemically manipulated to endoreduplicate using an aurora kinase inhibitor[20,26]. In these experiments, proteome composition also primarily changed with the ploidy-to-cell size ratio. As the surface area-to-volume ratio declines with increasing cell size regardless of changes in ploidy, we concluded that the relative surface area probably did not influence cell composition in the mammalian cells types that we measured.

After establishing that the ploidy-to-cell size ratio determined proteome composition in primary mouse hepatocytes, we next tested whether the same proteins were similarly affected by this ratio in mouse and human cells. To do this, we directly compared the size-scaling behavior of individual mouse and human proteins by matching them based on sequence orthology (DIOPT)[27]. Size-dependent changes to the mouse and human proteome were highly correlated (Fig. 1g and Supplementary Table 2). Moreover, differential size scaling in organelle protein content, a prominent signature of cell size[20], was the same in mice and human cells (Fig. 1h,i). The similarity of size-dependent proteome remodeling in these distinct cell types suggests that the ploidy-to-cell size ratio influences the proteome composition of most mammalian cells proliferating in culture.

Size-dependent changes in proteome composition are conserved

To test whether size-dependent proteome remodeling is conserved across eukaryotes, we turned to the single-celled organism budding yeast (*Saccharomyces cerevisiae*). We chose to examine budding yeast because of its genetic trackability and the approximately one billion years of evolutionary distance between yeast and mammals.

To measure how the proteome changes with budding yeast cell size, we generated different-sized yeast cells using orthogonal approaches and then measured proteome composition using a label-swapping, triple-SILAC (stable isotope labeling using amino acids in cell culture) workflow described previously[20] (Extended Data Fig. 2). First, we used mutant strains that have an average size that is either smaller (*whi5Δ*) or larger (*cln3Δ*) than a wild-type strain ('Size mutants'; Fig. 2a and Extended Data Fig. 3)[28]. Second, we used a strain that conditionally expresses a single G1 cyclin (Cln1) under the control of a β-estradiol-responsive promoter. In the absence of β-estradiol, these cells arrest in G1 because they lack expression of any of the endogenous G1 cyclins (*cln1Δcln2Δcln3Δ*)[29]. Biomass accumulates after washout at a rate that is initially near exponential (Fig. 2b). By sampling cultures that have been arrested for different lengths of time, we isolated cells of different mean sizes ('G1 arrest time'; Fig. 2a and Extended Data Fig. 3). Together, these two experimental systems span the physiological size range of budding yeast mother cells, which can grow to >200 fl as they age[30].

By manipulating yeast cell size in orthogonal ways, we found that the proteome remodeled continuously with changes in cell size (Fig. 2c). We calculated slope values to describe how the relative concentration of each measured protein changes as a function of cell size (Figs. 1b and 2d and Extended Data Fig. 3e). Size-dependent proteome changes were similar in both experimental systems (Fig. 2e and Supplementary Table 3), indicating that the proteome is continuously remodeled throughout an approximately fivefold size range in budding yeast (from *whi5Δ* to large G1-arrested cells). In fact, the concentrations of most proteins measurably changed with size (Extended Data Fig. 3h). The absolute abundance of a protein did not predict how its

Fig. 2 | Size-dependent changes in proteome composition are conserved from yeast to human cells. a, Orthogonal strategies to generate populations of different-sized budding. **b**, G1 arrest time course using conditional G1 cyclin expression. The arrows denote the time points measured by proteomics. Gray-scale colors represent the SILAC label. The dotted orange line represents the trajectory for exponential volume growth. **c**, PC1 of proteome changes across small, medium and large cells plotted against the mean cell volume. The dot size represents the mean cell volume. The colors represent the SILAC labels, which were swapped for three replicate experiments. All three experiments are plotted together. **d**, Top, relative intensities of SILAC peptide triplets plotted for three example proteins (H2B, RPB1, CIT1). Each dotted line represents an independent peptide measurement. Bottom, protein slope values from two replicate experiments (peptide measurement per protein >2). The *r* value denotes Pearson's correlation coefficient. exp, experiment. **e**, Correlation of protein slope values derived from the experimental systems in **a** ($n = 3$ for both systems). Only proteins with at least 12 independent measurements in both sets of experiments are shown (Supplementary Table 3). Core histone proteins are shown in red. The identity line is dashed. The blue dots are manually *x*-binned data and error bars represent the 99% CI. **f**, Size-scaling behavior of individual proteins strictly parsed by subcellular localization. Each dot represents a mean protein slope value ($n = 3$). The white dot represents the distribution's median. The *P* value is derived from two-sided Student's *t*-tests for the indicated comparisons. **g**, Annotation enrichment analysis using the protein slope values calculated from the two size separation strategies. Each dot is a significantly enriched annotation group (Supplementary Table 4). The dot size is determined by the number of proteins in each group and the position of the dot is determined by the mean slope value of the protein group. **h**, Correlation of mean protein slope values for budding yeast (G1 arrest time) and cultured human cells[20]. Proteins are paired by sequence orthology. Core histone proteins are shown in red, blue dots are evenly sized *x*-binned data and error bars represent the 99% CI. **i**, Distribution of slope values derived from budding yeast and human cells[20]. The top distribution considers only the proteins paired by orthology (plotted in **h**). **j**, Annotation enrichment analysis (as in **g**) using the orthology-paired human and yeast protein slope values (Supplementary Table 4). TCA, Tricarboxylic acid cycle.

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concentration changed with cell size (Extended Data Fig. 3f). As in mammalian cells, the proteome content of different organelles scaled differently with cell size (Fig. 2f). The size scaling of vacuolar proteins was consistent with size scaling of vacuolar volume[31]. Notably, although the total concentration of mitochondrial protein remained mostly constant (average protein slope of 0.07), individual mitochondrial proteins adopted a wide variety of scaling behaviors (s.d. of mitochondrial protein slope values of 0.25). Thus, although mitochondrial volume may scale in close proportion to cell volume[32,33], its protein composition changes. Two-dimensional (2D) annotation enrichment analysis[34] revealed a range of scaling behaviors across many different annotation groups (Fig. 2g and Supplementary Table 4). Our proteome measurements were in general agreement with what had been previously measured in excessively large, arrested cells[35]. Importantly, we also

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Independent variables	OLS regression (r^2)	Prediction (%)
Exp no.1 versus mean of no.2 and no.3	0.82	100
Protein localization only	0.12	15
mRNA slope	0.42	51
+ codon bias	0.45	54
+ protein turnover	0.50	60
Combined model	0.52	63

Fig. 3 | Transcriptional and post-transcriptional mechanisms underlie the size-scaling behavior of individual yeast proteins. a, Size-dependent changes in mRNA transcript concentration. The mRNA slopes were calculated in a manner analogous to the protein slopes (G1 arrest time). Transcript TPM values were used to estimate changes in transcript concentration. A correlation of two replicate experiments is shown (Supplementary Table 5). The blue dots are evenly sized *x*-binned data and error bars represent the 99% CI. **b**, The mean slope values for 3,529 proteins ($n = 3$)/transcripts ($n = 2$) are correlated. **c**, Linear regression models predicting protein slope values based on transcriptional and post-transcriptional variables. The protein localization model is based on binary association with a select set of subcellular compartments (as in Fig. 2f). Codon bias and protein turnover data were iteratively incorporated into a model derived from mRNA slopes. The combined model represents all the independent variables as well as subcellular localization. Correlation between biological replicates was used as a benchmark for the maximum predictive power. **d**, Correlation of mean protein slope (G1 arrest time, $n = 3$) with post-transcriptional model variables. Codon bias (left) and protein turnover (right, hours until 50% replacement) values were taken from the SGD[38] and a previous study[37], respectively. **e**, Comparison of protein slopes predicted from the combined linear model and mean protein slopes ($n = 3$) measured from G1-arrested cells.

confirmed that size-dependent proteome changes were not the result of auxotrophy in our SILAC-compatible yeast strains or the depletion of a limiting nutrient in the medium (Extended Data Fig. 4).

When grouped by subcellular localization, the size-dependent proteome changes in budding yeast were similar to those of mammalian cells (Figs. 1i and 2f). To quantitatively compare compositional changes in yeast and mammalian cells, we matched individual yeast and human proteins based on sequence orthology[27] and compared how their concentrations changed with cell size. The regression of protein slope values from orthology-matched yeast and human proteins produced a strongly significant correlation ($P < 0.0001$) (Fig. 2h and Supplementary Table 2). The degree to which the proteome composition changes with cell size is also similar in yeast and humans (Fig. 2i). The 2D annotation analysis of the orthology-matched protein slopes revealed similar size-scaling behaviors for most protein annotation groups. The most prominent difference between how the proteome scales with size in yeast and mammalian cells was in the proteins of the secretory network, which superscaled only in mammalian cells (Fig. 2j and Supplementary Table 4). Nevertheless, we found the conservation of proteome size scaling in yeast and human cells more striking than the differences.

Transcriptional regulation drives compositional change

After identifying widespread size-dependent changes in budding yeast proteome composition, we next sought to investigate the underlying molecular mechanisms. To determine whether size-dependent proteome changes are regulated transcriptionally, we performed RNA sequencing (RNA-seq) analysis on different sized cells and calculated mRNA slope values (Fig. 3a and Supplementary Table 5). The mRNA slopes strongly correlated with their corresponding protein slopes (Fig. 3b), indicating that size-dependent changes in proteome composition are largely the result of changes in gene expression. This finding is consistent with the few cases of differential scaling studied in-depth, Whi5 and histones, where protein subscaling was caused by mRNA subscaling[7,12,36].

In addition to the role of size-dependent changes in transcript concentration, we investigated whether post-transcriptional variables also contributed to proteome size scaling using linear models (Fig. 3c and Supplementary Table 5). We found that a protein's turnover[37] and codon bias[38] both correlated with the protein slope (Fig. 3d). Iterative incorporation of each parameter (mRNA slope, protein turnover and codon bias) improved the model with minimal collinearity. Using the correlation between biological replicates as a benchmark for the maximum size-dependent variation that any model could predict, we found that our composite model predicted 63% of this maximum size-dependent variance (Fig. 3e). Taken together, our linear models indicated that size-dependent proteome remodeling was driven both transcriptionally and post-transcriptionally.

Increasing cell size activates the general stress response

The environmental stress response (ESR), a prominent transcriptional program in budding yeast, has been associated with excessively large, arrested cells (>250 fl)[35,39]. When the ESR is activated, it triggers changes in the expression of around one-sixth of budding yeast genes[40,41]. To investigate whether ESR activation contributes to size-dependent proteome remodeling in cells closer to the physiological cell size range, we compared size-dependent proteome changes with those observed in response to several different stress conditions. This analysis revealed substantial overlap between size-dependent proteome remodeling and the compositional changes triggered by several acute stresses, including osmotic stress[42], starvation[43] and mammalian target of rapamycin (mTOR) inhibition (the present study), as well as a previously reported signature associated with slow steady-state growth (Fig. 4a)[44,45]. In addition to their correlation with size-dependent proteome changes, all these -omics datasets show strong crosscorrelation (Fig. 4a and Supplementary Table 6). Thus, all these perturbations trigger a response consistent with ESR activation.

That the proteomes of large cells correlate with the proteomes of stressed cells suggests that large cell size is a form of general stress. Proteins associated with the induced ESR (iESR) superscaled with cell size, whereas proteins associated with the repressed ESR (rESR) subscaled (Fig. 4b)[40]. In agreement with our observations with G1-arrested cells, other recent work also observed ESR activation in large arrested cells[35,39]. The activation of the ESR was not dependent on cell-cycle arrest because the ESR was also observed in asynchronously proliferating mutant strains (Fig. 4b). In fact, we found that the degree of ESR activation increased in almost linear proportion to cell volume across both asynchronously proliferating size mutants and G1-arrested cells (Fig. 4c, Extended Data Fig. 5a and Supplementary Table 6). Deletion of *MSN2* and *MSN4*, the two genes that encode the primary facilitators of the ESR[41], attenuated the superscaling of some but not most ESR-associated proteins (Fig. 4d and Extended Data Fig. 5b–e). ESR activation did not occur when increased cell size was accompanied by increased ploidy (Fig. 4e–f, Extended Data Fig. 5f–i and Supplementary Table 7), suggesting that this response is triggered by changes in genome concentration rather than a decrease in surface area:volume ratio. It is interesting that the deletion of *WHI5*, which reduces cell size, decreased ESR activation relative to wild-type cells (Fig. 4e,f and Extended Data Fig. 5f–i).

Large cell size explains some age-associated phenotypes

The induction of a stress response on cell enlargement may have important implications for replicative aging. Over the course of their lifespans, budding yeast mothers produce ~25 daughters, growing progressively larger before permanently exiting the cell cycle[46]. We therefore hypothesized that some age-associated proteome changes could be explained by the increased size of old yeast mothers.

To test the hypothesis that increased cell size drives some of the compositional changes associated with old yeast cells, we measured how the proteome changes during yeast aging and compared these data with the proteomic signature of increased cell size. To do this, we combined a biotin–streptavidin affinity purification procedure with a genetic selection scheme known as the 'mother enrichment program' to isolate aged yeast (Fig. 5a,b)[46]. This allowed us to isolate and quantify the proteomes of aged mother cells and their daughters. In concordance with previous observations, we observed substantial differences between the proteomes of old and young yeast, correlating most strongly with a recently published study (Extended Data Fig. 6d)[47]. These data correlated well with the proteomic signature of increased cell size (Fig. 5d, Extended Data Fig. 6c and Supplementary Table 8) despite differences in strain background. Moreover, most age-associated changes in the proteome were also present in the daughters of old cells (Extended Data Fig. 6a), which have a very similar lifespan to the daughters of young cells. These observations suggest that some age-associated proteome changes are explained by the increased size of old mother cells. These include sentinel age-associated changes, such as an apparent decrease in histone proteins (Fig. 5c and Extended Data Fig. 6b), often referred to as 'nucleosome loss'[48]. Our data suggest that this phenomenon arises from the increased size of older yeast rather than an age-associated loss of existing histones.

If some age-associated proteome changes are a consequence of increased cell size, to what extent are the pathologies associated with aging, most notably senescence, the result of large cell size? To test this, we G1-arrested yeast for different periods of time and then induced them to re-enter the cell cycle (Fig. 5e). We found that, when our G1-arrested cells surpassed a size threshold (~230 fl), their viability sharply declined (Fig. 5e, bottom-most panel). This decline in viability is consistent with a similar experiment done using a temperature-sensitive, cell-cycle arrest[35]. The size range around which a large fraction of cells permanently lost the ability to re-enter the cell cycle corresponded to the upper size range of old haploid yeast mother cells[30]. This observation supports the hypothesis that cell size is an important determinant of budding yeast lifespan, rather than simply a correlate[30,49].

Increasing cell size elicits a starvation-like phenotype

Thus far our experiments suggest that genome concentration (that is, ploidy-to-cell size ratio) determines eukaryotic proteome composition and size-dependent compositional changes in yeast are, in part, explained by the upregulation of the ESR. However, it is unclear how a change in genome concentration would result in a change in cell composition. One phenotype tightly associated with the ESR in budding yeast is a slow growth rate. Budding yeasts growing in a nutrient-limited environment strongly upregulate the ESR, even when in the steady state[44]. It is interesting that the specific growth rate (mass added per unit mass) of yeast or human cells declines as cell size increases (Fig. 2b and Extended Data Fig. 3b)[50–52]. Thus, the genome may become limiting for cell growth in large cells (Fig. 6a) and size-dependent changes in proteome composition may reflect this decline in relative growth rate. Consistent with this model, the growth defects and proteome remodeling associated with cell enlargement are averted when an increase in size coincides with an increase in ploidy (Figs. 1e and 5g and Extended Data Fig. 5i)[20,26,35,51].

To test our model that genome dilution mimics a limiting nutrient (Fig. 6a), we attempted to mimic size-dependent proteome remodeling by attenuating the growth rate (Fig. 6b). We acutely inhibited cell growth in proliferating yeast (the present study) and human cells[51] using the highly specific mTOR inhibitor, rapamycin. Size-dependent proteome remodeling in both yeast and human cells strongly resembled that which occurred after acute mTOR inhibition (Fig. 6c and Supplementary Table 9), with the notable exception of the histones and other chromatin-associated proteins (Fig. 6c)[51]. As the size-dependent proteome remodeling resembled the compositional changes induced by rapamycin, we next tested whether changes in cell-growth signaling coincided with cell enlargement using phosphoproteomics

Fig. 4 | Increasing cell size activates the general stress response. a, Matrix showing the crosscorrelation of size-dependent proteome remodeling with stress-associated proteome/transcriptome changes. The correlation values are drawn from 1,101 proteins/mRNAs shared between all datasets in the grid (Supplementary Table 6). For the Murphy et al.[43] dataset, protein concentration ratios were calculated from cultures in either the log or the lag phases of the diauxic shift. For Soufi et al.[42], concentration ratios were calculated before and after exposure to 400 mM NaCl. Concentration ratios were calculated before and after a 150-min treatment of 1 μM rapamycin. The chemostat datasets anti-correlate with the protein slope because the 'growth rate slope' reported from Brauer et al.[44] reflected no stress/stress ratio orientation whereas the other datasets had a stress/no stress orientation. **b**, Distribution of mean protein slope values ($n = 3$) for the genes associated with the r- and iESR[40,44]. The rESR consists of ribosomal protein (RP) and ribosomal biogenesis (RiBi) clusters. See Supplementary Table 3 for a list of all ESR-associated genes. The white dot represents the distribution's median. The *P* values were derived from two-sided Student's *t*-tests. NS, Not significant. **c**, Size-dependent changes in the concentrations of ESR-associated proteins across an approximately fourfold range of budding yeast cell sizes (Supplementary Table 6). The mean cell size increases from left to right (*whi5Δ* to the final time point of the G1 arrest). Two replicates for each mean size are shown as paired columns in the heatmap. **d**, Correlation of size-dependent proteome changes in cells with and without Msn2/4. ESR proteins with baseline expression in small WT cells dependent on Msn2/4 (Extended Data Fig. 5d) are highlighted in blue. Histones are highlighted in red. **e**, A strategy to generate different-sized yeasts with a similar ploidy-to-cell size ratio. The relative surface area decreases as the cell size increases, regardless of changes in ploidy (Extended Data Fig. 5f,g). **f**, Mean concentration ratio ($n = 2$) from a label-swapped SILAC comparison of the indicated strains growing in asynchronous cultures (Supplementary Table 7). Gray cumulative distribution represents all measured proteins. The iESR proteins and histone proteins are highlighted in blue and red, respectively.

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(Fig. 6d and Extended Data Fig. 7). To assess how cell signaling changes during cell enlargement, we grouped phosphorylation events according to the co-regulation modules defined by Leutert et al.[53]. In brief, Leutert et al. identified 2,191 highly dynamic phosphorylation events. They grouped these sites into 29 distinct modules based on their co-regulation dynamics in response to various perturbations. Our analysis identified the module corresponding to TORC1–Sch9 signaling as the most downregulated in large cells (Fig. 6e,f and Supplementary Table 10). Taken together, we conclude that increasing cell size elicits a starvation-like phenotype in yeast and mammalian cells (Fig. 6g)

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Fig. 5 | The large size of old yeast explains some age-associated phenotypes. **a**, A strategy to enrich for replicatively aged yeast based on the mother enrichment program[46]. After incubating labeled cultures for different periods of time, the proteomes of young (0 h), middle-aged (24 h) and old (48 h) yeasts were measured using TMT. **b**, Example fluorescent and phase images of young, middle-aged and old yeasts. Bud scars are fluorescently labeled (WGA-FITC). Images illustrate the increased size of aged budding yeast mothers. **c**, Relative concentrations of core histone proteins in young, middle-aged and old yeasts. Each dot corresponds to one of three biological replicate experiments. **d**, Correlation of age-associated and size-dependent proteome remodeling. Concentration ratio (old:young) is the average of three replicate experiments (Supplementary Table 8). The protein slope is the mean value from G1 arrest time experiments (Fig. 2). Only proteins with >3 independent peptide measurements in each dataset are considered. Histone proteins are highlighted in red, blue dots are evenly sized *x*-binned data and error bars represent the 99% CI. The *r* value denotes Pearson's correlation coefficient. **e**, Spot assay showing the decline in proliferative capacity in very large cells. The mean cell size at the time of spotting on +β-estradiol plates is shown in femtoliters. Each spot represents a 3× dilution in cell number (from left to right). Each plate was imaged after 40 h of growth at 30 °C.

and this phenotype may arise from growth-related limitations of genome concentration.

Intrinsic and extrinsic determinants of proteome composition

That cell enlargement elicited a starvation-like phenotype raised the question as to how changing growth conditions, through changing the carbon source, would influence how proteome composition changes with cell size. To examine this, we first measured the proteome of yeast growing asynchronously on a nonfermentable carbon source (ethanol/glycerol) and compared it with the proteome of yeast growing on glucose (Fig. 7a and Extended Data Fig. 8a,b). Yeast utilizing a nonfermentable carbon must rely on respiration to catabolize carbon and, as a result, grow ~50% slower. We calculated a concentration ratio (respiration:fermentation) for 4,162 yeast proteins (Fig. 7a, Extended Data Fig. 8d and Supplementary Table 11). As expected, the fraction of the proteome dedicated to respiratory metabolism and the iESR was higher in yeast utilizing the nonfermentable carbon source (Fig. 7b). Almost half of the proteins measured in our analysis changed in concentration by twofold or more between carbon sources. The propensity of high copy number proteins (first abundance quartile) to change in concentration was the same as the low copy number proteins (fourth abundance quartile) (Fig. 7c and Extended Data Fig. 8c). In other words, the proteome is extensively remodeled when shifting between these two carbon sources. Yet, despite the large difference in baseline proteome composition between fermenting and respiring yeast, the way in which the proteome remodeled with cell size within these two metabolic states was remarkably similar (Fig. 7d, Extended Data Fig. 8e,f and Supplementary Table 3). Some of the protein groups that were most upregulated in respiring yeast (Fig. 7b) were no longer superscaling with cell size when the yeasts were grown in ethanol/glycerol medium (Fig. 7e,f). Although the iESR superscaled with size regardless of carbon source, its degree of superscaling was attenuated in ethanol/glycerol medium. This may suggest that the baseline upregulation of these protein groups in yeast growing in ethanol/glycerol is distinct from the superscaling behavior present in glucose. Overall, these results suggest that proteome composition is determined intrinsically via cell size in a way that is mostly separable from extrinsic environmental cues, although the size-scaling behavior of certain protein groups may depend on growth conditions.

Discussion

It was long thought that the concentrations of individual proteins remain mostly constant as cells grow larger, in part because total protein amount scales in direct proportion to cell size[54]. Recent proteomic analyses have overturned the previous paradigm by identifying widespread size-dependent changes in the concentrations of individual proteins[20,21]. In the present study, we identify conserved principles for size-dependent proteome remodeling by extending our analysis to evolutionarily divergent eukaryotes. We found that, in mammalian and yeast cells, the proteome remodels continuously as cells grow larger. These findings are important because they provide a simple and universal rationale for the regulation of cell size, namely, the further a cell deviates from its target size, the more compositionally different it becomes and the worse it performs its most important functions.

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Fig. 6 | Increasing cell size elicits a starvation-like phenotype in mammalian and yeast cells. a, Schematic illustrating how cell growth could be limited by the genome. The dotted line represents exponential biomass accumulation. As genome concentration decreases, the relative growth rate declines (Fig. 2b and Extended Data Fig. 3b). A decline in the relative growth rate is not observed in large polyploid cells[26]. **b**, Schematic illustrating experimental logic. If size-dependent proteome remodeling relates to size-dependent changes in growth rate, then does acute growth inhibition in proliferating cells produce similar proteome changes? **c**, Correlation of protein slope values and proteome remodeling in response to acute growth inhibition of proliferating cells (saturating dose of rapamycin for 150 min and 48 h in budding yeast and human cells, respectively). Concentration ratios are the average of two replicate experiments. The correlation in human cells is re-plotted from published work[51]. The blue dots are manually *x*-binned data and the error bars represent the 99% CI. The *r* value denotes Pearson's correlation coefficient. **d**, A phosphoproteomic approach to determine whether changes in cell signaling coincide with size-dependent proteome remodeling (see Extended Data Fig. 7 for methodology). **e**, Phosphopeptide measurements grouped based on co-regulation modules described by Leutert et al.[53]. The median $\log_2$(ratio) for all phosphopeptide measurements within each module is plotted. Module no. 12 corresponds to phosphorylation events downstream of mTORC1–Sch9 signaling (see Supplementary Table 10 for all phosphopeptide measurements). **f**, Cumulative distribution for all phosphopeptide measurements (gray) and phosphopeptides that map to co-regulation module 12 (green). **g**, Schematic illustrating how genome dilution by cell growth elicits a 'starvation-like' signaling and proteome remodeling in eukaryotic cells.

A stoichiometric relationship between some proteins and DNA

In every experiment where we manipulated cell size in eukaryotes, the relative change in the concentration of histone proteins matched the expected change in the concentration of the genome. To achieve this, transcription of histone loci is tightly coupled to the S phase in an amount independent of cell size[12,36]. In addition, histones produced in excess because of delays in DNA replication are degraded[55], resulting in protein amounts that are always proportional to the amount of DNA. In addition to the histone proteins, the concentrations of several other DNA-associated proteins (for example, cohesion, condensin and chromatin-remodeling complexes) also aligned with changes in genome concentration (Fig. 2f)[51], although it is unclear whether they are subject to the same mechanisms of regulation as histones. The maintenance of a strict stoichiometry between the genome and tightly DNA-bound proteins, such as histones, may prevent an excess of these proteins in the chromatin. Notably, the maintenance of protein:DNA stoichiometry is probably important because the overexpression of histone proteins can be toxic[56].

Is cell enlargement stressful?

Perhaps the most striking set of superscaling proteins in budding yeasts are those associated with the general stress response (Fig. 4b,c). The notion that cell enlargement is intrinsically stressful is consistent with observations in cultured mammalian cells linking excessive

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Fig. 7 | External environment has a large effect on proteome composition, but only a small effect on how the proteome changes with cell size. **a**, Proteome comparison of yeast growing asynchronously in medium containing glucose or ethanol/glycerol as a carbon source. Relative concentration ratios (respiration:fermentation) for individual proteins measured in two replication experiments are plotted (Supplementary Table 11). The *r* value denotes Pearson's correlation coefficient. The data density is shaded. **b**, Fraction of the proteome dedicated to the indicated GO term in asynchronously proliferating cultures. Proteome fraction is calculated from the summed intensity of all the peptides from all proteins possessing the indicated GO term divided by the total peptide ion intensity. Each dot represents a biological replicate. **c**, Distribution of relative concentration changes (respiration:fermentation) for proteins with different expression levels. Protein abundance was separated into quartiles using summed intensity for all peptide measurements for a given protein. The first and fourth quartiles represent the 25% most and least abundant proteins in the dataset, respectively. The *x* axis represents the mean ratio from the experiments in **a**. **d**, Correlation of protein slope values derived from yeast growing in ethanol/glycerol- and glucose-containing media. Core histone proteins are shown in red. The glucose-derived protein slopes are from the G1 arrest time experiment in Fig. 2. The same experimental system was used to generate the ethanol/glycerol-derived protein slope values. Each protein slope is the mean value of three replicate experiments. Only proteins with >3 independent measurements in each dataset are considered (see Supplementary Table 3 for all measurements). The blue dots are *x*-binned data and the error bars represent the 99% CI. **e**, Violin plots depicting the distribution of protein slope values for the indicated annotation groups. Each protein slope is the mean value of three replicate experiments. The white dot represents the distribution's median value. *P* values are derived from two-sided Student's *t*-tests. **f**, The 2D annotation enrichment analysis using the protein slope values derived from glucose and ethanol/glycerol-metabolizing yeast. Each dot is an annotation group and the position of the dot is determined by the mean slope value of the proteins in the group (Supplementary Table 4). RSC, chromatin structure remodeling.

cell enlargement to senescence, the state of irreversible (or durable) cell-cycle arrest in which cells fail to divide in the presence of mitogens or oncogenic stimuli[57]. Mounting evidence suggests that cell enlargement promotes senescence-associated proteome changes and cell-cycle exit[20,21,35,58–61], but the mechanism by which this happens is unclear. As in yeast, excessive cell size could trigger a mammalian stress response leading to increased expression of the Cdk inhibitors p16 or p21, which are associated with senescence[57]. Indeed, multiple independent groups have now reported that p21 is upregulated with cell enlargement in various human cell lines[20,21,62–65]. One potential source of p21 induction is the increased susceptibility to DNA damage in large cells[20,62], which may, in part, result from the replication stress caused by the subscaling of DNA replication and repair proteins[66] or an inability to initiate DNA repair[62]. Other proposed sources of stress during cell enlargement include cytoplasmic dilution[35] and osmotic stress[64]. Consistent with links between cell enlargement and senescence identified in vitro, recent work has shown that the proliferative potential of only slightly enlarged hematopoietic stem cells is severely compromised in vivo[67].

In the case of budding yeasts, stress induction is also tightly linked to decreases in the cellular growth rate[44]. Surprisingly, we found size-dependent changes in ESR expression even in cases where the cellular growth rate was not changing, for example, the ESR is upregulated in an asynchronously proliferating *cln3Δ* strain (Fig. 4g), which has a doubling time close to wild-type (Extended Data Fig. 3a). Similarly, we observed a reduction of ESR expression in the smaller *whi5Δ* strains (Fig. 4g), which also proliferate at rates similar to wild-type cells (Extended Data Fig. 3a). ESR induction is therefore probably caused by increased cell size rather than by cell-cycle arrest because the degree of ESR induction is directly proportional to differences in size regardless of whether cells were arrested in the cell cycle (Fig. 4b). It is possible that even wild-type yeast experiences some basal-level stress due to

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Fig. 8 | Cell size is an intrinsic determinant of cell composition in growing cells. a, Illustration of how growth rate (top) and proteome composition (bottom) change with cell size. The absolute growth rate (biomass production per time) increases with cell size. The specific growth rate (growth rate per unit biomass) is constant across the cell size mutants (Extended Data Fig. 3a), but begins to decline in large G1-arrested cells (Extended Data Fig. 3b). Regardless of the changes in growth dynamics, the proteome remodels continuously with increasing cell size. The *x* axis is not drawn to scale. **b**, Working model depicting how the proteome composition of growing cells has both intrinsic and extrinsic determinants.

their size because the concentration of ESR-associated proteins can be decreased through mutations such as *whi5Δ*, which reduce cell size but do not affect proliferation rates (Extended Data Fig. 3a). On the other hand, it is possible that the size-dependent activation of the ESR may not be directly driven by stress itself. Rather, the size-dependent activation of the ESR could represent an evolved mechanism to passively prepare smaller daughters for the stress that will come as they become progressively larger with age. Regardless of its causality, a recent study demonstrated that ESR induction in large, arrested yeast cells reduces the decline in their viability[39]. This suggests that the ESR response in progressively larger cells is beneficial.

Genome dilution attenuates cell growth like a limiting nutrient

The induction of general stress response has generally been linked to changes in the cellular growth rate in budding yeast. Attenuating cell growth by limiting the availability of any single nutrient results in the upregulation of ESR transcripts[44]. Moreover, the proteome remodeling that occurs in response to acute stress is similar to how the yeast proteome changes when cells are starved or grown at steady state in carbon-limiting medium (Fig. 4a). It is unclear how the general stress response is coupled to changes in growth rate, but this coupling is functional in certain scenarios because slow-growing cells are more resistant to certain types of acute stress[68].

In the present study, we observed that the ESR is upregulated in large cells and that this is the result of the dilution of the yeast genome eliciting a starvation-like proteome phenotype (protein slope versus diauxic shift; Fig. 4a). Indeed, in both yeasts and mammals, size-dependent proteome changes are strikingly similar to those that occur after cell growth has been acutely inhibited (Fig. 6c)[51]. The observation that increasing cell size promotes a starvation-like proteome phenotype suggests that cell growth is intrinsically limited by the genome in a manner analogous to a limiting nutrient. The changing growth dynamics of enlarging yeasts could also explain the predictive power of post-transcriptional regulation in our linear models (Fig. 3c). As cells grow larger and relative growth rate declines, a protein's stability and the translational efficiency of its mRNA transcript could influence its final concentration. The starvation-like proteome changes in large cells may also explain the allometric size scaling of mitochondrial membrane potential, which has been described previously[69,70].

Importantly, the widespread proteome changes that occur with increasing cell size in budding yeast precede a measurable decline in their specific growth rate (Fig. 8a). This contrasts with the current models for how different proteome 'sectors' are reallocated when cells are grown in different nutrient conditions, which describes a linear relationship between proteome composition and the cellular growth rate. For example, the proteome sector allocated to protein synthesis decreases (as a fraction of the total proteome) when cells are grown on poorer carbon sources in both yeast and bacteria[45,71–73]. However, that we see proteome remodeling in large cells even before the growth rate declines shows that the relationship between proteome composition and growth dynamics is probably more complex than the simple linear relationships described for ribosomes[74].

A model for how cell size determines proteome composition

In the present study, we established how genome dilution, through changes in the ploidy-to-cell size ratio, impacts proteome composition in yeast and mammalian cells (Fig. 8b). First, many DNA-associated proteins, such as histones and chromatin-remodeling complexes, maintain a stoichiometric relationship with the amount of DNA in a cell and are therefore diluted as cells grow larger. Second, dilution of the genome appears to restrict cell growth in a manner analogous to a limiting nutrient, because large cell size elicits a starvation-like proteome phenotype. Importantly, a similar relationship between genome concentration and proteome composition was recently described in *Escherichia coli*[75]. We anticipate that future work will identify the underlying molecular mechanisms controlling these distinct behaviors and give insight into how cell size-dependent proteome remodeling affects cell physiology.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41594-024-01353-z.

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Methods

Primary hepatocyte isolation, culture and FACS sorting

Primary hepatocytes from Rosa26-CreER2;FUCCI2 mice were isolated using two-step collagenase perfusion[76]. Liver perfusion medium (Thermo Fisher Scientific, cat. no. 17701038), liver digest medium (Thermo Fisher Scientific, cat. no. 17703034) and hepatocyte wash medium (Thermo Fisher Scientific, cat. no. 17704024) were used for this process. After isolation, cells were washed 3× with hepatocyte wash medium. Cells were then plated in a six-well plate precoated with collagen I (50 μg ml^{-1}) at a density of 200,000 per well. The culture medium contained 3 μM CHIR99021 (Peprotech), 25 ng ml^{-1} of epidermal growth factor (Peprotech), 50 ng ml^{-1} of hepatocyte growth factor (Peprotech) and 100 ng ml^{-1} of tumor necrosis factor-α (Peprotech) in basal medium. The basal medium contained William's E medium (Gibco), 1% Glutamax (Gibco), 1% nonessential amino acids (Gibco), 1% penicillin–streptomycin (Gibco), 0.2% normocin (Invitrogen), 2% B27 (Gibco), 1% N2 supplement (Gibco), 2% fetal bovine serum (Corning), 10 mM nicotinamide (Sigma-Aldrich), 1.25 mM *N*-acetylcysteine (Sigma-Aldrich), 10 μM Y27632 (Peprotech) and 1 μM A83-01 (Tocris). The culture medium was refreshed every other day. Cells were passaged via trypsinization using TrypLE (Thermo Fisher Scientific).

For FACS, primary hepatocytes were trypsinized and incubated with Hoechst (Thermo Fisher Scientific, cat. no. 62249) at 10 μg ml^{-1} and 37 °C for 30 min. Cells were then washed with hepatocyte wash medium (see above) and sorted using a Flow Cytometer (BD FACSAria II) based on their FUCCI cell-cycle marker and DNA staining (Extended Data Fig. 1). Only G1 (with high mCherry-Cdt FUCCI signal and low Venus-Geminin FUCCI signal) hepatocytes with the same ploidy were sorted. Side scatter (SSC-A) was used to indicate size[17] and three size bins were gated to sort small, medium and large cells. Cells, 10,000–20,000, were sorted for each population. After sorting, 10% of the sorted cells were used to measure cell size using a Z2 Coulter counter (Beckman) and the rest of the cells were lysed in radioimmunoprecipitation lysis buffer supplemented with protease inhibitors and phosphatase inhibitors. Proteins were then denatured, reduced, alkylated and precipitated as described in the liquid chromatography–tandem MS (LC–MS/MS) sample preparation section.

Budding yeast growth

All yeasts were grown in synthetic complete minimal medium (US Biological, cat. no. Y2030) with standard nitrogen, carbon (2% dextrose) and amino acid supplements unless otherwise indicated. Yeasts grown for SILAC analysis were cultured in synthetic minimal medium lacking lysine and arginine. These cultures were supplemented with light, intermediate or heavy versions of lysine (115 μM) and arginine (200 μM) (Cambridge Isotope Laboratories). The light (Agr0; Lys0) version of the medium contained L-arginine and L-lysine built with normal ^{12}C and ^{14}N isotopes; the intermediate (Arg6; Lys4) version had L-arginine containing six ^{13}C atoms and L-lysine containing four deuterium atoms; the heavy (Arg10; Lys8) version had L-arginine containing six ^{13}C and four ^{15}N atoms and L-lysine containing six ^{13}C and two ^{15}N atoms. To ensure complete labeling, the cells were grown in SILAC medium for approximately ten doublings before starting an experiment.

G1 arrest-time experiments were performed using either the strain yML127 (*ARG1*$^+$*LYS4*$^+$) or the strain yML129 (*arg1Δlys4Δ*). These strains require β-estradiol (final concentration of 20 nM; Sigma-Aldrich, cat. no. E2758) to proliferate. To initiate the G1 arrest time course, β-estradiol was removed from the culture by centrifugation, followed by a single, pre-warmed medium wash. Alternatively, cells were collected on a 0.2-μm disposable filter, then washed with pre-warmed medium. Washed cells were then resuspended in medium without β-estradiol (t = 0 min of the arrest time course). After 2 h and 15 min in (−)β-estradiol medium, >95% of the yeasts are unbudded. Mean cell size was determined using a Z2 Coulter counter (Beckman).

Large-scale isolation of mothers

The Mother Enrichment Program[46] was used to isolate old budding yeast mothers. Strain YDJ 1334 was grown to exponential phase and 6×10^8 cells were distributed between two sterile 15-ml conical tubes. The cells in each tube were then spun down, washed with phosphate-buffered saline (PBS), resuspended in 500 μl of PBS and labeled for 30 min with 3 mg of EZ-Link Sulfo-NHS-LC-Biotin (in dark, gentile agitation and at room temperature). Biotin-labeled cells were washed 3× with 1 ml of PBS and then split into three aliquots. One aliquot was stored immediately at −80 °C (young yeast) and the other two were each used to inoculate 1 l of YPD culture, one that grew for 24 h (middle-aged mothers) and the other that grew for 48 h (old mothers). β-Estradiol (Sigma-Aldrich, cat. no. E2758) was then added to 1 μM in each liquid culture for the duration of the incubation period. Strain YDJ 1334 contains genetic modifications that allow for the selective killing of newly born daughter cells in the presence of β-estradiol.

To isolate mothers after the 24- or 48-h incubation period, each 1 l of culture was spun down, washed 2× with PBS and resuspended in a 50-ml conical tube with 10 ml of PBS. Resuspended cells were then mixed with 500-μm MACS streptavidin magnetic beads (Miltenyi Biotec, cat. no. 130-074-101) and mutated for 30 min (in dark at room temperature). Streptavidin-labeled cells were washed twice more with PBS and then resuspended in 80 ml of PBS. Streptavidin-labeled mothers were then captured on LS+ Positive Selection Columns (Miltenyi Biotec, cat. no. 130-042-401). In brief, four columns were used for each time point (24 and 48 h). Each column was placed in a magnet quadro MACS (Miltenyi Biotec) and rinsed with 5 ml of PBS. Cells were loaded into each column 8 ml at a time (20 ml in total for each column) and then washed 2× with 8 ml of PBS. Column-bound cells were then eluted by gravity flow (14 ml of PBS) after removal from the magnet. Our results were compared with other published datasets[47,77] in Extended Data Fig. 6.

LC–MS/MS sample preparation

SILAC. See Supplementary Table 12 for a detailed accounting of all proteomic experiments. Yeast cultures were pelleted by centrifugation or collected on a 0.2-μm disposable filter. Pellets were resuspended in 300 μl of yeast lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.2% Tergitol, pH 7.5 + a cOmplete ULTRA Tablet) with 700 μl of glass beads. Lysis was performed at 4 °C in a Millipore Fastprep24 (4 cycles with the following settings: 6.0 m s^{-1}, 4× 40 s). Cell lysates were cleared by centrifugation at 12,000*g* for 5 min at 4 °C. Protein concentration was quantified using a Pierce BCA Protein Assay Kit (product no. 23255). Equal amounts of protein from each SILAC-labeled lysate were mixed. The mixed lysates were then denatured/reduced in 1% sodium dodecylsulfate (SDS) and 10 mM dithiothreitol (15 min at 65 °C), alkylated with 5 mM iodoacetamide (15 min at room temperature) and then precipitated with three volumes of a solution containing 50% acetone and 50% ethanol (on ice for 10 min). Proteins were re-solubilized in 2 M urea, 50 mM Tris-HCl and 150 mM NaCl, and then digested with TPCK-treated trypsin (50:1) overnight at 37 °C. Trifluoroacetic acid (TFA) and formic acid were added to the digested peptides for a final concentration of 0.2% (pH ~2). Peptides were desalted with 50-mg Sep-Pak C18 columns (Waters).

TMT. Lysis, denaturation, reduction and precipitation for tandem mass tagging (TMT) analysis was the same as described above for SILAC analysis with the exception that the lysis buffer and working solution of iodoacetamide used Hepes instead of Tris. Our method for TMT labeling was adapted from Zecha et al.[78] and the Thermo TMT-10plex or TMTpro 16plex Isobaric Label Reagent Set Protocol. In brief, ethanol:acetone-precipitated proteins were resuspended in 100 mM triethylammonium bicarbonate (TEAB) and digested overnight with TPCK trypsin (50:1). After digestion, the peptide concentration

was ~1 μg μl^{-1} in 100 mM TEAB for all samples. Then, 20 μg of peptide was labeled using 100 μg of Thermo TMT10plex or TMTpro 16plex in a reaction volume of 25 μl for 1 h. The labeling reaction was quenched with a final concentration of 0.5% hydroxylamine for 15 min. Labeled peptides were pooled, acidified to pH ~2 using drops of 10% TFA and desalted with a Sep-Pak 50-mg C18 column.

High-pH reverse-phase fractionation

SILAC- or TMT-labeled peptides were fractionated using a Pierce High pH Reversed-Phase Peptide Fractionation kit. The eight default fractions were either injected separately or pooled back into four fractions (1–5, 2–6, 3–7 and 4–8). Dried peptides were reconstituted in 0.1% TFA. Peptide concentrations were determined using a nanodrop before injection.

LC–MS/MS data acquisition

SILAC. Desalted SILAC-labeled peptides were analyzed on a Fusion Lumos mass spectrometer (Thermo Fisher Scientific) equipped with a Thermo EASY-nLC 1200 LC system (Thermo Fisher Scientific). Peptides were separated by capillary reverse-phase chromatography on a 25-cm column (75-μm inner diameter, packed with 1.6-μm C18 resin, cat. no. AUR2-25075C18A, Ionopticks). Peptides were introduced into the Fusion Lumos mass spectrometer using a 125-min stepped linear gradient at a flow rate of 300 nl min^{-1}. The steps of the gradient are as follows: 3–27% buffer B (0.1% (v:v) formic acid in 80% acetonitrile) for 105 min, 27–40% buffer B for 15 min, 40–95% buffer B for 5 min and finally maintaining at 90% buffer B for 5 min. Column temperature was maintained at 50 °C throughout the procedure. Xcalibur software (v.4.4.16.14, Thermo Fisher Scientific) was used for the data acquisition and the instrument was operated in data-dependent mode. Advanced peak detection was enabled. Survey scans were acquired in the Orbitrap mass analyzer (Profile mode) over the range 375–1,500 *m/z* with a mass resolution of 240,000 (at *m/z* 200). For MS1, the normalized AGC target (%) was set at 250 and maximum injection time was set to 'Auto'. Selected ions were fragmented by higher-energy collisional dissociation with normalized collision energies set to 31 and the fragmentation mass spectra were acquired in the ion trap mass analyzer with the scan rate set to 'Turbo'. The isolation window was set to the 0.7-*m/z* window. For MS2, the normalized AGC target (%) was set to 'Standard' and maximum injection time to 'Auto'. Repeated sequencing of peptides was kept to a minimum by dynamic exclusion of the sequenced peptides for 30 s. Maximum duty cycle length was set to 1 s.

TMT. Instrumentation and ionization for TMT analysis was the same as the SILAC experiments described above. Peptides were introduced into the Fusion Lumos mass spectrometer using a 180-min stepped linear gradient at a flow rate of 300 nl min^{-1}. The steps of the gradient are as follows: 6–33% buffer B (0.1% (v:v) formic acid in 80% acetonitrile) for 145 min, 33-45% buffer B for 15 min, 40–95% buffer B for 5 min and maintain at 90% buffer B for 5 min. The column temperature was maintained at 50 °C throughout the procedure. Xcalibur software (v.4.4.16.14) was used for the data acquisition and the instrument was operated in data-dependent mode. Advanced peak detection was disabled. Survey scans were acquired in the Orbitrap mass analyzer (centroid mode) over the range 380–1,400 *m/z* with a mass resolution of 120,000 (at *m/z* 200). For MS1, the normalized AGC target (%) was set at 250 and maximum injection time was set to 100 ms. Selected ions were fragmented by collision-induced dissociation (CID) with normalized collision energies of 34 and the tandem mass spectra were acquired in the ion trap mass analyzer with the scan rate set to 'Rapid'. The isolation window was set to the 0.7-*m/z* window. For MS2, the normalized AGC target (%) was set to 'Standard' and maximum injection time to 35 ms. Repeated sequencing of peptides was kept to a minimum by dynamic exclusion of the sequenced peptides for 30 s. The maximum duty cycle length was set to 3 s. Relative changes in peptide concentration were determined at the MS3 level by isolating and fragmenting the five most dominant MS2 ion peaks.

Spectral searches for TMT and SILAC

All raw files were searched using the Andromeda engine[79] embedded in MaxQuant (v.2.4.2)[80]. In brief, a three-label SILAC search was conducted using MaxQuant's default Arg6/10 and Lys4/8 labels. For TMT searches, a Reporter ion MS3 search was conducted using 10plex or TMTpro isobaric labels. For both TMT and SILAC searches, variable modifications included oxidation (M) and protein amino-terminal acetylation. Carbamidomethyl (C) was a fixed modification. The number of modifications per peptide was capped at five. Digestion was set to tryptic (proline blocked). For SILAC experiments, peptides were not 'Re-quantified' and MaxQuant's match-between-runs feature was not enabled. The database search was conducted using the UniProt proteome—Yeast_UP000002311_559292 or Mouse_UP000000589_10090. The minimum peptide length was seven amino acids. The 1% false recovery rate (FDR) was determined using a reverse decoy proteome.

Phosphopeptide enrichment

Roughly 2 mg of SILAC-labeled peptide was desalted before phosphopeptide enrichment (as described above). Phosphopeptides were enriched using a High Select Fe-NTA Phosphopeptide Enrichment Kit (Thermo Fisher Scientific, cat. no. A32992). After elution from the spin column, phosphopeptides were fractionated using the High-pH reverse-phase fractionation described above.

Trapped ion mobility time-of-flight MS

Desalted SILAC-labeled phosphopeptides were analyzed on a trapped ion mobility time-of-flight (timsTOF)-SCP mass spectrometer (Bruker) equipped with a Thermo EASY-nLC 1200 LC system (Thermo Fisher Scientific). Peptides were separated by capillary reverse-phase chromatography on a 25-cm column (75-μm inner diameter, packed with 1.7-μm C18 resin, captive spray). Peptides were introduced to the timsTOF-SCP using a 74-min stepped linear gradient at a flow rate of 100 nl min^{-1}. The steps of the gradient are as follows: 3–27% buffer B (0.1% (v:v) formic acid in 80% acetonitrile) for 52 min, 27–40% buffer B for 8 min, 40–95% buffer B for 2 min and finally maintain at 90% buffer B for 12 min. Column temperature was maintained at 50 °C throughout the procedure.

Samples were analyzed by PASEF-dda with a duty cycle of 1.03 s comprising one MS1 survey for every five PASEF MS2 ramps and a mass range between 100 *m/z* and 1,700 *m/z*. The trapped ion mobility spectrometry (TIMS) device ramp/accumulation time was set to 166 ms with a 100% duty cycle and a mobility range of 0.7–1.3 1/K0. Precursor ions from the survey scan were selected using an adjusted isolation width of 2 *m/z* below 700 *m/z* and 3 *m/z* above 800 *m/z* precursor mass-to-charge value in the quadrupole that aligns with the anticipated TIMS elution time relative to the ion mobility value. Fragmentation was performed by CID with collisional energy interpolation between 42 eV at 0.65 1/K0, 31.92 eV at 0.8 1/K0, 36.96 eV at 1 1/K0, 42 eV at 1.2 1/K0, 47.04 eV at 1.4 1/K0 and 51.24 eV at 1.6 1/K0. Active exclusion of precursors was released after 0.2 min. Precursor repetitions were set to a target intensity of 20,000 and a threshold of 500.

Phosphoproteome analysis

A three-label SILAC search was conducted using MaxQuant's default Arg6/10 and Lys4/8 labels (as described above). Variable modifications were set for phosphorylation at serine, threonine and tyrosine. Phosphopeptide-level analysis was performed using the evidence.txt output table. Phosphosite-level information from the Phospho (STY) Sites.txt output table was imported into the evidence.txt using an in-house script. A unique phosphopeptide measurement was defined by concatenating the 'Modified sequence', 'Charge' and 'Fraction' to the phosphosite ('UniqID' column of Supplementary Table 10). Phosphopeptide ratios for three replicate experiments were determined using

the ion intensity columns in the evidence table. See Supplementary Table 12 for SILAC channel information for each experiment. CDK sites highlighted in Extended Data Fig. 7d were taken from Holt et al.[81].

RNA extraction and RNA-seq

RNA extraction and RNA-seq were performed by Azenta (poly(A) enrichment, Standard RNA-Seq Analysis package). To calculate the mRNA slope values, the relative changes in an mRNA's transcript per million (TPM) value were used as a proxy for its relative change in concentration.

Peptide quantification

SILAC. Our SILAC analysis pipeline had been employed previously[20]. We used the peptide feature information in MaxQuant's evidence.txt output file. Peptide triplets (each row in the evidence.txt table) are assigned to a protein based on MaxQuant's 'Leading razor protein' designation. For each peptide triplet, the fraction of ion intensity in each SILAC channel was calculated by dividing the 'Intensity L/M/H' column by the 'Intensity' column. SILAC channels were normalized by adjusting the fraction of ion intensity in each channel by the median for all measured peptides (Extended Data Fig. 2b,c). After normalization, the relative signal difference between the SILAC channels for each peptide triplet was plotted against the normalized cell size (Fig. 1b). A unique peptide measurement is defined by the peptide sequence, modification state, charge state and fraction number. See Supplementary Table 12 for SILAC channel information for each experiment.

For each unique peptide measurement, we calculated its slope as follows:

$Y_{1,2,3}$ = relative signal in each SILAC channel (order based on labeling orientation)
Average size = (mean volume of small bin + mean volume of medium bin + mean volume of large bin)/3
x_1 = (mean volume of small size bin)/average size
x_2 = (mean volume of medium size bin)/average size
x_3 = (mean volume of large size bin)/average size.

Based on the expectation that our experimental conditions would not result in large, nonlinear changes in protein expression, we excluded peptide triplets with three data points that did not loosely fit a linear regression line. Linear regressions on the triplets were performed using np.polyfit in Python. Regressions with a mean squared error >0.2 were excluded. As this filtering step improved the overall data quality (Extended Data Fig. 2e), we concluded that our filtering method mostly excluded peptide triplets contaminated by analytical interference or near the noise floor.

Individual peptide measurements were consolidated into a protein-level measurement using Python's groupby.median. Peptides with the same amino acid sequence that were identified as different charge states or in different fractions were considered independent measurements. We summarized the size-scaling behavior of individual proteins as a slope value derived from a regression (similar to what is described above for individual peptides) and each protein slope value is based on the behavior of all detected peptides.

For a given protein, we calculated its slope as follows:

y_i = relative signal in the ith SILAC channel (median of all peptides measured for this protein)
x_i = same normalized cell size x_i as for the peptide slope calculations above.

The protein slope value was determined from a linear fit to the log(transformed data) using the equation:

$$\log_2(y) = \text{Slope} \times \log_2(x).$$

Variables were log(transformed) so that a slope of 1 corresponds to an increase in protein concentration that is proportional to the increase in volume and a slope of −1 corresponds to 1/volume dilution. Pearson's r and P values for correlation analyses were calculated using scipy's pearsonr module in Python.

TMT. Our TMT analysis pipeline uses the peptide feature information in MaxQuant's evidence.txt output file. Each row of the evidence.txt file represents an independent peptide and its corresponding MS3 TMT measurement. Peptides without signal in any of the TMT channels were excluded. TMT peptide measurements were assigned to a protein based on MaxQuant's 'Leading razor protein' designation. For each peptide measurement, the fraction of ion intensity in each TMT channel was calculated by dividing the 'Reporter ion intensity' column by the sum of all reporter ion intensities. TMT channels were normalized by adjusting the fraction of ion intensity in each channel by the median for all measured peptides (similar to the SILAC normalization in Extended Data Fig. 2b,c). After normalization, the relative signal difference between the TMT channels for each peptide triplet was plotted against the normalized cell size for each of the bins of isolated G1 cells. Slope values in Extended Data Fig. 2e,f were derived in a manner analogous to the slope values calculated in the SILAC experiments. See Supplementary Table 12 for TMT channel information for each experiment.

Regression analysis

Regression plots used throughout the manuscript were generated using seaborn.regplot package in Python. Grayed dots represent all data points in the regression analysis. For each plot, the x variable is grouped into a defined number of evenly sized (not necessarily evenly spaced) bins. Each bin is represented by a dark-blue dot. When indicated, the spacing of the bins along the x axis was manually determined (manually set). Each dot represents a bin's mean y-axis value and its error bar is the 99% confidence interval (CI) for the y coordinate. Unless indicated otherwise, the displayed r value represents Pearson's correlation coefficient. Pearson's r and P values for correlation analyses were calculated using SciPy's pearsonr module in Python.

Orthology analysis

Human ortholog pairs were retrieved using the DRSC Integrative Ortholog Prediction Tool (DIOPT) found at https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl (ref. 27). Yeast or mouse proteins were used as the 'input' species and humans were set as the 'output'. For budding yeast, the systematic gene name was used for the 'search term', and for mice the UniProt ID was used. We only considered ortholog pairs with a DIOPT 'weighted score' of >10 for our analyses. Once mouse or yeast proteins had been matched with a human ortholog protein, we imported protein slope values derived from a human RPE-1 cell line[20].

Two-dimensional annotation enrichment analysis

Annotation enrichment analysis was performed as described previously[34]. The protein annotation groups were deemed significantly enriched and plotted if the Benjamini–Hochberg FDR <0.02. The position of each annotation group on the plot is determined by the enrichment score (S). The enrichment score is calculated from the rank-ordered distribution of protein slope values:

$$S = 2(R_{\text{group}} - R_{\text{remaining proteins}})/n$$

where R_{group} and $R_{\text{remaining proteins}}$ are the average ranks for the proteins within an annotation group and all remaining proteins in the experiment, respectively, and n is the total number of proteins. Highlighted annotation groups were manually curated. All significantly super- and subscaling annotation groups can be found in Supplementary Table 4.

Principal component analysis
PCA analysis was performed in Python using the sklearn package. A data frame was created that contained individual proteins as rows with columns corresponding to the relative protein concentration in each SILAC or TMT channel (obtained from the median of all peptide measurements for a given protein).

Protein annotations
For 2D annotation enrichment plots, the default gene ontology (GO) GOMC, GOMF and GOCC annotations were used. For all other plots, protein localization annotations were based on UniProt columns named 'Subcellular location [CC]' or 'Protein names'. Protein localization was strictly parsed so that each annotated protein belongs to only one of the designated groups. Proteins with two or more of the depicted annotations were ignored (except for the 'Cytoplasm/Nucleus' category, which required both a nuclear and a cytoplasmic annotation). ESR-associated genes were annotated based on previous work[40].

OLS linear regression model
Multiple linear regression analyses were performed using the statsmodels module in Python. The prediction of protein slope was based on the 2,375 proteins shared across the protein turnover[37], mRNA slope and protein slope datasets (at least 4 peptides per protein). Codon bias values for each gene were taken from 'Coding Region Translation Calculations' on the Protein tab of the *Saccharomyces* Genome Database (SGD). Each independent variable (mRNA slope, protein turnover and codon bias) was standardized by subtracting all values by the dataset's mean and then dividing by the dataset's s.d. The subcellular localization variable was based on UniProt's 'Subcellular location [CC]' annotations and entered as a binary value for each compartment (1 if a protein possessed an annotation and 0 if it did not). Only subcellular compartments that provided nonredundant predictive power were ultimately included in the model. A constant value was added to the regression equation using the add_constant function in statsmodels. The benchmark for predictive accuracy (prediction (%)) was set as the correlation between biological replicate experiments (protein slope from Exp no. 1 versus the average slope from Exp nos. 2 and 3). See Supplementary Table 5 for input data related to the ordinary least squares regression models.

Cell-surface area measurement
Cells were grown to early log(phase) in synthetic complete medium + 2% glucose and gently sonicated before being loaded on to a glass slide. The 2 × 2 binned phase contrast images were taken with a Zeiss Axio Observer Z1 microscope using a plan-apo ×63/1.4 numerical aperture oil immersion objective. Cell-volume measurements were performed using the existing algorithm in Cell-ACDC[82] v.1.4.7. Cell-ACDC was used to segment the cell boundaries with the convolutional neural network algorithm YeaZ[83]. The volumes of mother and bud pairs were added together.

Assuming that each mother or bud is shaped like a prolate ellipsoid with polar radius, c, and equatorial radius, a, the surface area S of each ellipsoid can be calculated using the following equation:

$$S = 2\pi a^2 + 2\pi \frac{ac}{\sqrt{1-\frac{a^2}{c^2}}} \arcsin\sqrt{1-\frac{a^2}{c^2}}$$

Cell-ACDC measures the major ($2c$) and minor ($2a$) axis lengths of an ellipse the approximate shape of each mother or bud body. These outputs were used with the equation above to measure the cell-surface areas. The surface areas of mother and bud pairs were added together.

Statistics and reproducibility
For all box and violin plots, the box region represents the median and interquartile range (IQR), with tails extending to 1.5× the IQR. All P values are two sided and were calculated using the default settings of the scipy.stats.ttest_ind module in Python. For SILAC-based proteomic analyses, metabolic labels were swapped between replicate experiments to reduce technical noise from culture conditions and analytical interference during data acquisition. To enhance the presentation of protein-level quantification throughout the manuscript, datasets were frequently filtered to exclude proteins with one or few peptide-level measurements. In all cases, the complete datasets can be found in the form of supplementary tables.

No statistical method was used to predetermine sample size and the experiments were not randomized. For some proteomic experiments not all TMT channels were used for data analysis (Supplementary Table 12). As no data in this manuscript were manually scored, the investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
The UniProt proteome database (https://www.uniprot.org) was used as a reference to search mass spectral data. The data used to generate all the figures are provided as supplementary tables. All raw proteomics datafiles are available on PRIDE (accession no. PXD052786). All raw mRNA-seq files are available on the Gene Expression Omnibus (accession no. GSE269091).

Code availability
The code used to process proteomics data and calculate the protein slope value is available at https://github.com/mikechucklanz/Yeast-proteomics-processing-and-slope-calculation (ref. 84).

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Author contributions

M.C.L. designed and carried out yeast-related experiments, except for Fig. 7d (performed by L.H.) and Fig. 5 (performed by D.F.J. and I.Z.). M.C.L. prepared samples for MS analysis and acquired MS data. M.C.L. and F.M. maintained the performance of the mass spectrometers. M.C.L. performed all data analyses. S.Z. derived the hepatocyte primary cells and FACS-isolated the cell populations measured in Fig. 1. M.P.S. and M.C.L. performed mRNA-seq for Fig. 3. M.P.S. constructed the SILAC yeast strains. J.K. calculated the surface:volume ratio for diploid and haploid yeast strains. M.C.L. and J.M.S. wrote the paper. J.M.S. and J.E.E. supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Supplement for Fig. 1. (a) Stained liver section illustrating the heterogeneity of hepatocyte ploidy. Mononucleated cells are diploid and binucleated cells are tetraploid. See methods for detailed description of primary cell isolation and culturing. **(b)** FACS scheme to isolate G1 phase primary hepatocyte cells of different sizes and ploidy. A fluorescent FUCCI cell cycle reporter was used to identify G1 phase cells. After gating for G1 cells, 2n and 4n G1 cells were differentiated using a DNA stain. Diploid and tetraploid G1 cells were separated by size using the side scatter parameter. **(c)** Example images of post-sorted primary hepatocytes stained with Hoechst. The mononucleated cell is diploid and the binucleated cell is tetraploid. Sorted populations were lysed and peptides were quantified using MS3-TMT proteomics (Fig. 1; methods).

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Extended Data Fig. 2 | See next page for caption.

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Extended Data Fig. 2 | Supplement for Fig. 2. (a) Budding yeast of different sizes were metabolically labeled in cell culture and subjected to proteomic analysis. Strategies to separate cells by size are described in Fig. 2 and Extended Data Fig. 3. See methods for processing steps prior to mass spec data acquisition. **(b)** After cell lysis, protein concentrations were quantified. An equal amount of protein from small-, medium-, or large-cell size populations (each possessing a unique metabolic SILAC label) were mixed. Loading error from the mixing process was normalized as described in the methods. Rather than normalize L/H and L/M SILAC ratios separately, we normalize all three channels together so that the values in our dataset represent relative changes across all cell sizes. Data normalization from a poorly mixed hypothetical example experiment is depicted. **(c)** For each individual peptide triplet, we determined the fraction of the triplet's total ion intensity present in each SILAC channel. The distributions of these fractions were then adjusted by the median (see methods for a complete description of the normalization process). Data from the hypothetical example in (b) is depicted. **(d)** Peptide slope values are calculated from a linear regression of the relative ion intensity in each SILAC channel and mean cell size. Mean cell size was determined by Coulter counter prior to mixing and lysis. The mean squared error was used to track the linear fit of each peptide triplet regression. **(e)** Correlation of peptide slopes calculated from replicate experiments before and after applying a filter for mean squared error (MSE). 146,492 unique peptide measurements were identified in two replicate experiments ("G1 arrest time" from Fig. 2). A unique peptide measurement is defined by the peptide sequence, modification state, charge state, and pre-fractionation fraction number. Loosely filtering peptide triplets by MSE increased the correlation between biological replicate experiments.

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Extended Data Fig. 3 | Supplement for Fig. 2. (a) Cell cycle distribution and steady-state growth rate is unaffected by the different SILAC labels. Cell size mutants have doubling times similar to wild type (~90 min) when growing asynchronously in synthetic complete media with 2% glucose. **(b)** Growth behavior before and after G1 arrest in different SILAC media. The curved trajectory indicates a declining specific growth rate ~200 minutes after washout. Measurements of volume and optical density were collected in parallel on the same cultures. Y-axis is relative to 135 min, which is when ~95% of cells are in G1 phase. **(c)** The attainment of differentially sized cells was confirmed using a Coulter counter. Color gradients correspond to replicate cultures grown with light, medium, and heavy SILAC labels. **(d)** PC1 vs PC2 plotted for both orthogonal experimental systems. **(e)** Illustration of the protein slope calculation from relative changes in peptide concentration and cell size for both orthogonal experimental systems. See methods for a detailed explanation of the protein slope calculation. **(f)** To determine if protein abundance predicts size-scaling behavior, the individual proteins were grouped into abundance quartiles (depicted in the left plot). We only considered the ion intensity from the "small" cell SILAC channel in each of three G1 arrest time replicate experiments. The right plot depicts the distribution of mean slope values (G1 arrest time, n=3) for the proteins in each abundance quartile. Box region represents the median and interquartile range (IQR). Tails extend to 1.5x the IQR. **(g)** Correlation of protein slopes calculated from the two orthogonal experimental systems. Each slope value is the mean of three replicates for both systems. For the left panel, the mean volume measurements from the cell size mutants (C) were used to calculate the protein slope. For the right panel, the mean volume measurements from (C) were weighted by the relative DNA content shown in (A). Blue dots are x-binned data and error bars represent the 99% confidence interval. The "adjusted" plot is shown in Fig. 2. **(h)** Heatmap depicting the relative concentrations of ~4000 budding yeast proteins in the G1 arrest time experiment shown in (c) and (d). Proteins are ordered from top to bottom by descending protein slope value.

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Extended Data Fig. 4 | See next page for caption.

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Extended Data Fig. 4 | Supplement for Fig. 2. (a) Experimental scheme to test whether SILAC-related mutations or culture density affects size-dependent proteome changes. The indicated samples were labeled with 10-plex TMT. A protein slope value was calculated in a manner similar to Fig. 2d. Here, TMT-MS3 reporter ions were used to quantify relative changes in protein concentration rather than MS1 SILAC triplet ions. **(b)** Cell size distributions determined by a Coulter counter. Time points of the G1 arrest time course are differentially colored. **(c)** During the G1 arrest time course, culture flasks were repeatedly diluted with pre-warmed media to maintain a constant culture density. **(d)** Principal component analysis of the proteome measurements of the G1 arrested cells. The 1st principal component is plotted against mean cell volume. Dot size represents mean cell volume. Colors represent the replicate experiments in the different strain backgrounds depicted in (a). **(e)** Correlation of protein slope values calculated in the *arg4Δlys1Δ* and *ARG4 LYS1* strain backgrounds from (a). Each slope was calculated from a single replicate experiment. Blue dots are x-binned data and error bars represent the 99% confidence interval. r value denotes the Pearson correlation coefficient. **(f)** Correlation of protein slope values calculated using TMT with the protein slope measurements calculated using SILAC (G1 arrest time, Fig. 2). TMT-calculated slope is the mean of the two axes plotted in (e). Blue dots are x-binned data and error bars represent the 99% confidence interval. r value denotes the Pearson correlation coefficient.

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Extended Data Fig. 5 | See next page for caption.

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Extended Data Fig. 5 | Supplement for Fig. 4. (a) Heatmap of relative protein concentration changes across different cell sizes. Each size is represented by two replicate columns. Size mutants correspond to asynchronous *whi5Δ*, WT, and *cln3Δ* cultures. G1 arrest time corresponds to four time points after G1 arrest taken at 1-hour intervals (using the genetic systems described in Fig. 2). **(b** and **c)** Coulter counter measurements of (b) *MSN2 MSN4* and (c) *msn2Δmsn4Δ* G1-arrested cells. Biological replicate experiments are differentially shaded. **(d)** Proteins whose expression is dependent on *msn2Δmsn4Δ* are denoted in blue. Proteins highlighted in blue are the same set depicted in Fig. 4d. Msn2/4-dependency was defined as decrease in concentration of > 1.6-fold. **(e)** Fig. 4d is re-plotted here for reference. The same genes identified in (d) as Msn2/4-dependent are shown in blue. **(f)** Relative surface area (*that is*, surface area-to-volume ratio) for individual yeast cells was calculated from wide-field images of asynchronously proliferating cells. Yeast mothers and buds are assumed to be prolate ellipsoids. By measuring the polar (major axis) and equatorial (minor axis) radii, the surface area *S* and volume *V* of each ellipsoid was calculated (see methods). **(g)** Relative surface area ($\mu m^2 / \mu m^3$) measurements for individual cells of the indicated strain background. Average surface area-to-volume ratio for each strain is marked by the dashed horizontal line. **(h)** Coulter counter measurements of the indicated strains. Biological replicate experiments are differentially shaded. SILAC channels were swapped for replicate experiments to maximize measurement accuracy. **(i)** Correlation of protein slope values (G1 arrest time) with the concentration ratios calculated from the binary comparison of the indicated strains. Protein slope values are the average from three replicate experiments (see Fig. 2). Each concentration ratio is the average of two SILAC label-swapped replicate experiments. Core histone proteins are shown in red. Blue dots are x-binned data and error bars represent the 99% confidence interval. r value denotes Pearson correlation coefficient.

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Extended Data Fig. 6 | See next page for caption.

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Extended Data Fig. 6 | Supplement for Fig. 5. (a) Correlation of $\log_2$ (old / young) concentration ratios derived from young and old budding yeast mothers and their daughter cells. Each concentration ratio is the average from three replicate experiments. Blue dots are x-binned data and error bars represent the 99% confidence interval. **(b)** Relative concentrations of core histone proteins in young, middle-aged, and old yeast mothers. Each dot corresponds to 1 of 3 biological replicate experiments. Measurements of the daughters of young, middle-aged, and old mothers is depicted in Fig. 5. **(c)** Correlation of age-associated and size-dependent proteome changes. Log_2 concentration ratio (old / young) is calculated from mother cells. Protein slope value is from the G1 arrest time experiment in Fig. 2. Measurements of the daughters of young, middle-aged, and old mothers is depicted in Fig. 5. Concentration ratios are the average from three replicate experiments. Blue dots are x-binned data and error bars represent the 99% confidence interval. r value denotes Pearson correlation coefficient. **(d)** Correlation grid containing size- and age-associated proteomics data. Protein slope values (Fig. 2), $\log_2$ (old / young) concentration ratios for daughters (Fig. 5) and mothers (b), were cross correlated with similar measurements from the literature. Numbers and colors denote Pearson correlation value for indicated datasets.

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Extended Data Fig. 7 | Supplement for Fig. 6. (a) Schematic outlining a phosphoproteomic approach to determine whether changes in cell signaling coincide with size-dependent proteome remodeling. **(b)** The attainment of differentially-sized cells was confirmed using a Coulter counter. Color gradients correspond to replicate cultures grown with light, medium, and heavy SILAC labels. **(c)** Correlation of phosphopeptide ratios calculated from two of the three label-swapped replicate experiments. Each phosphopeptide measurement is colored based on the size-scaling behavior of the protein that harbors the phosphorylation event (*that is*, its protein slope). The correlation plot illustrates that the main source of variance in the phosphoproteomic data (Large / Small) is changes in the concentrations of the proteins harboring the phosphorylation sites. **(d)** Large changes in cell cycle-related signaling between asynchronous and arrested yeast. Cumulative distribution of all phosphopeptide ratios (gray) and phosphopeptides that harbor CDK1-dependent phosphorylation events (blue)[75]. **(e)** Phosphopeptide ratios (Large / Small) were corrected using the protein slope value (Fig. 2d). This correction reduces the variance in the phosphoproteome data set. Cumulative distribution of all phosphopeptide ratios before (red) and after (gray) a correction is applied to mitigate protein-level change in concentration. The gray distribution is depicted in Fig. 6f.

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Extended Data Fig. 8 | See next page for caption.

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Extended Data Fig. 8 | Supplement for Fig. 7. (a) Proteome comparison of budding yeast utilizing fermentable or non-fermentable carbon sources. Four label-swapping biological replicate experiments were initially performed. Two of these experiments were then pre-fractionated for deeper proteome analysis. The media mixtures used in the comparison were synthetic complete with 2% dextrose (Glucose) and synthetic complete with 1% ethanol and 2% glycerol (Ethanol/glycerol). **(b)** Principal component analysis of all 4 biological replicate experiments. The same plot is shown twice. Coloring highlights the source of variance for PC1 and PC2. **(c)** Protein abundance does not influence the degree to which a protein's concentration changes between growth conditions. For each individual protein, a crude estimation of copy number (summed peptide intensity) was used to bin the proteome by abundance quartiles. One of the two replicate experiments was used to calculate summed ion intensity for each protein. Box region represents the median and IQR. Tails extend to 1.5x the IQR. **(d)** Correlation of SILAC ratios for every unique peptide measurement shared between two biological replicate experiments. A unique peptide measurement is defined by the peptide sequence, modification state, charge state, and fraction number. To filter out peptide measurements that were contaminated by analytical interference, peptides that produced a reciprocal measurement between SILAC-swapped replicates were excluded (bottom plot). **(e)** G1 arrest time course (as described in Fig. 2) was performed in synthetic complete media supplemented 1% ethanol and 2% glycerol as a carbon source. The length of the G1 arrest time course was extended due to the slower growth rate in ethanol/glycerol media. Size separation was confirmed using a Coulter counter. Light-, medium-, and heavy-labeled yeast are differentially shaded. **(f)** Principal component analysis of the proteome measurements on small-, medium-, and large-sized cells grown in ethanol/glycerol media. The 1st principal component is plotted against mean cell volume. Dot size represents mean cell volume. Colors represent the SILAC label. SILAC labeling orientation for small, medium, and large cells was swapped for three replicate experiments. All three replicate experiments are plotted together.

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Extended Data Table 1 | Strains list

Strain	Short name	Full Genotype
ML1	Haploid W303	MATa, *leu2-3,112* , *trp1-1* , *can1-100* , *ura3-1* , *ade2-1* , *his3-11,15*
ML223	W303_SILAC	MATa, *W303, lys1Δ::HphNT1* , *arg4Δ::NatNT2* , *ADE+* , *CAN1+*
ML123	W303_SILAC_whi5Δ::KanMX	MATa, *W303, lys1Δ::HphNT1* , *arg4Δ::NatNT2* , *ADE+* , *CAN1+* , *whi5Δ::KanMX*
ML125	W303_SILAC_cln3Δ::KanMX	MATa, *W303, lys1Δ::HphNT1* , *arg4Δ::NatNT2* , *ADE+* , *CAN1+* , *cln3Δ::KanMX*
ML127	*cln1,2,3Δ* BEp-CLN2	MATa , *cln1Δ* , *cln2Δ* , *cln3::LEU2* , *LexAPr-CLN1-LEU2* , *his3::LexA-ER-AD-HIS3*
ML129	cln1,2,3Δ BEp-CLN2 (SILAC)	MATa , *cln1Δ* , *cln2Δ* , *cln3::LEU2* , *LexAPr-CLN1-LEU2* , *his3::LexA-ER-AD-HIS3*, *lys1Δ::HphNT1* , *arg4Δ::NatNT2*
ML217	cln1,2,3Δ BEp-CLN2 msn2Δmsn4Δ	MATa , *cln1Δ* , *cln2Δ* , *cln3::LEU2* , *LexAPr-CLN1-LEU2* , *his3::LexA-ER-AD-HIS3* , *msn2Δ::KanMX* , *msn4Δ::HphMX*
ML239	DIPLOID_W303_SILAC	MATa/MATalpha, *W303/W303, lys1Δ::HphNT1/lys1Δ::HphNT1* , *arg4Δ::NatNT2/arg4Δ::KanNT2* , *ADE+/ADE+* , *CAN1+/CAN1+*

Budding yeast strains used in this study.

nature portfolio

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☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Xcalibur (v4.4.16.14), FACSDiva (v9.0), COULTER Z2 AccuComp (v3.01)
Data analysis	Maxquant (v2), Python (v 3.7.17), Jupyter Notebook (v5.7.10)

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Uniprot proteome database (https://www.uniprot.org/) was used a reference to search mass spectral data. The data used to generate all the figures is provided as supplemental tables. All raw proteomics datafiles are available on PRIDE (^^^). All raw mRNA sequencing files are available on GEO (^^^).

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	SILAC experiment were all done in duplicate or triplicate. TMT labeled experiments were done in duplicate as is standard in the field. All quantitative comparisons were done within a multiplexed set (TMT or SILAC).
Data exclusions	For some experiments, not all TMT channels were used (as described in S12). This is because multiple independent experiments were sometimes multiplexed together. We felt some of these experiments were not relevant for this publication.
Replication	We performed all experiments in at least duplicate and compared results to ensure reproducibility. In many cases, we plot a correlation between our replicate experiments in the main figure panels. We used different methods to manipulate cell size to ensure our conclusions are robust.
Randomization	Three different SILAC labels were used to quantify three different sized yeast populations, so we conducted three replicate experiments where each size population was grown and quantified using a different SILAC label.
Blinding	Blinding was not required because data generation did not rely on manual measurement or scoring.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
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Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)	Primary mouse hepatocytes generated by us from profused liver
Authentication	N/A
Mycoplasma contamination	primary cell lines were used immediately following generation and not test for mycoplasma
Commonly misidentified lines (See ICLAC register)	N/A

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	Rosa26-CreER2; FUCCI2 (3 weeks old)
Wild animals	No wild animals were used in the study
Reporting on sex	All haploid yeast used in this study were mating type a
Field-collected samples	We did not collect samples in the field
Ethics oversight	The study did not require ethical oversight

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

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Methodology

Sample preparation	*Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.*
Instrument	BD FACSAria II
Software	Manufacturers software
Cell population abundance	10-20,000 cells were sorted for each population

Gating strategy	Described in Extended Data Figure 1

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