Non-random distribution of vacuoles in Schizosaccharomyces pombe

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Abstract
A central question in eukaryotic cell biology asks, during cell division, how is the growth and distribution of organelles regulated to ensure each daughter cell receives an appropriate amount. For vacuoles in budding yeast, there are well described organelle-to-cell size scaling trends as well as inheritance mechanisms involving highly coordinated movements. It is unclear whether such mechanisms are necessary in the symmetrically dividing fission yeast, Schizosaccharomyces pombe, in which random partitioning may be utilized to distribute vacuoles to daughter cells. To address the increasing need for high-throughput analysis, we are augmenting existing semi-automated image processing by developing fully automated machine learning methods for locating vacuoles and segmenting fission yeast cells from brightfield and fluorescence micrographs. All strains studied show qualitative correlations in vacuole-to-cell size scaling trends, i.e. vacuole volume, surface area, and number all increase with cell size. Furthermore, increasing vacuole number was found to be a consistent mechanism for the increase in total vacuole size in the cell. Vacuoles are not distributed evenly throughout the cell with respect to available cytoplasm. Rather, vacuoles show distinct peaks in distribution close to the nucleus, and this preferential localization was confirmed in mutants in which nucleus position is perturbed. Disruption of microtubules leads to quantitative changes in both vacuole size scaling trends and distribution patterns, indicating the microtubule cytoskeleton is a key mechanism for maintaining vacuole structure.

1. Introduction
Vacuoles in yeasts are homologous to mammalian lysosomes, play critical roles in cellular biochemical pathways, and are largely responsible for protein degradation and nutrient storage [1]. Vacuole/lysosome size and morphology are both regulated via numerous mechanisms, and abnormal size and abundance of lysosomes are hallmarks of a family of developmental disorders including Batten’s disease [2].

Previous research in the budding yeast Saccharomyces cerevisiae demonstrate that there is a consistent scaling relationship between cell and organelle size, including nuclei, [3, 4] mitochondria, [5] and vacuoles [6, 7]. In other words, as cells grow in size, organelle size also increases at a roughly constant proportion. Since budding yeast grow by asymmetric budding, the site of bud growth is distinct from the location of organelles, and the cell actively transports vacuoles from mother to the newly forming bud via inheritance mechanisms [8]. Inheritance is critical to maintain proper vacuole structure and for buds to contain an appropriate amount of vacuoles after cell division. Mutant budding yeast strains with defective vacuole inheritance exhibit altered vacuole-cell size scaling, [7] cell-cycle delays, [9] and fitness defects [10]. This raises the question, are vacuole size
and distribution regulated in a different fashion in cells that have different morphologies and division patterns?

In the fission yeast species *Schizosaccharomyces pombe*, wild-type cells exhibit a rod-like morphology, grow at the cell tips, and divide symmetrically at the cell mid-line to create two equally-sized daughter cells. Vacuoles in fission yeast tend to be more numerous than in budding yeast [1, 11]. This provides an interesting comparison to mammalian lysosomes that are also present in cells in high copy numbers and exhibit dynamics in their distribution patterns [12]. Because of the contrasts in cell and vacuole morphologies with budding yeast, we believe fission yeast provide an intriguing model to find distinct methods to partition vacuoles during cell division. One simple model is that the stochastic inheritance of numerous vacuoles in the cell proportional to available cytoplasmic space would be sufficient to ensure that daughter cells could receive roughly equal amounts of organelles from the mother [11, 13]. Such a scenario would not require active positioning of vacuoles. However, it is also possible that vacuoles localize to specific areas depending on the needs of the cell.

We are interested in how fission yeast cells regulate vacuole size and localization. In this manuscript, we begin by investigating if, as it has been observed for other organelles such as the nucleus, [14] there is a scaling trend between cell size (measured by length) and vacuole size (measured by number, surface area and volume). By studying cells with altered size, we ask how robust vacuole surface area or volume scaling trends are, and we elucidate possible mechanisms for maintaining overall vacuole size. To determine how these symmetrically-dividing cells can ensure proper inheritance of vacuoles during division, we investigate the distribution pattern of vacuoles along the cell axis and the partitioning of vacuoles between the two cell halves on either side of the nucleus. Further experiments utilize mutants and drugs that break the symmetry of cell growth and nuclear positioning and ask how vacuole distribution is impacted.

### 2. Materials and methods

#### 2.1. Cell strains and culture

Table 1 provides a list of strains used in this study, their genotypes, and their relevant phenotypes. Cells were grown in YESS rich media at 30 °C for non-temperature sensitive or 25 °C for temperature-sensitive mutants (*wee1-ts*, *cdc25-ts*). Cells were grown through at least two doublings to log phase. At this point, non-ts strains were labeled and imaged, and temperature-sensitive strains were shifted to 37 °C for 2 h before imaging. For experiments examining the effect of microtubule depolymerization, wild-type cells were grown to log phase. A 100x stock of thiamendazole in DMSO was added to the cell culture (Sigma-Aldrich) to a working concentration of 100 μg ml⁻¹, and the culture was incubated at 30 °C for 2 h before imaging.

#### 2.2. Cell labeling

Cell cultures were incubated with the vacuole membrane dye FM-4-64 at a working concentration of 16 μM for 15–30 min. Excess dye was removed by two cycles of pelleting cells by centrifugation, then resuspension in an equal volume of fresh media. Cell cultures were then incubated at 30 °C for a final chase period of 45 min. For additional labeling of the vacuole lumen, CDCFDA was also added to cell culture to a working concentration of 10 μg ml⁻¹, and incubated and washed as with FM-4-64. Cells were mounted on coverslips treated with 1 mg ml⁻¹ Concanavalin A for imaging.

#### 2.3. Imaging microscopy

Images were taken using a Zeiss Cell Observer equipped with a Yokogawa spinning disk head, 100x/1.4NA objective (Zeiss), and a Photometrics QuantEM:512SC EMCCD camera. Ti mutants were imaged inside a temperature-controlled stage at 35 °C, while non-ts cells were imaged at room temperature. Using these instruments, multiple z-stacks of individual cells were acquired at 0.2 μm intervals.

### Table 1. Strains used in this study.

| Strain | Relevant mutation | Genotype | Phenotype | Average cell length ± S.D. (μm) | N |
|--------|-------------------|----------|-----------|-------------------------------|---|
| SP-175 | Wild-type         | h⁻ 972   | Normal, full dataset | 9.8 ± 1.6 | 198 |
| SP-204 | wee1-ts          | h⁺ wee1-50 | Subset for shape model | 10.0 ± 1.5 | 31 |
| SP-205 | cdc25-ts         | h⁺ cdc25-32 ade6 ·M210 ura4-D18 leu1-32 | Premature entry into mitosis, smaller cells | 5.7 ± 1.4 | 46 |
| FC-1338| rsp1-1           | rsp1-1 cut11-GFP | Delayed cytokinesis, longer cells | Not measured | N/A |
| FC-1794| pom1Δ            | pom1Δ rcl1-GFP | Misplaced nucleus | 10.5 ± 1.9 | 32 |
| FC-1901| rga4Δ            | h⁺ rga4::ura4⁺ leu 1-32 ura4Δ-18 | Altered cell growth polarity | 9.4 ± 2.9 | 28 |
| mnl2Δ  | mnl2Δ            | h⁺ mnl2::KANMX4 ade6-M210 ura4-D18 leu1-32 | Altered cell growth polarity | 9.6 ± 1.4 | 60 |
|        |                   |          | Homolog to nucleus-vacuole junction protein in budding yeast | 10.7 ± 1.4 | 131 |
nominal spacing using both brightfield transmitted light modes and fluorescence excitation at the appropriate wavelengths for the dyes used. Excitation light was delivered from lasers at 488 nm (CDCFDA) and 561 nm (FM-4-64), and emission light was collected through band-pass filters with wavelength ranges of 525/50 nm (CDCFDA) and 629/62 nm (FM-4-64).

2.4. Basic image analysis

Representative images of the vacuoles in the yeast strains used in this study are shown in figure 1. Images were further processed using a combination of ImageJ and MATLAB platforms. A rolling-ball, background-removal algorithm was applied to reduce excess noise in fluorescent z-stacks. Vacuole centroids were then manually annotated in three dimensions by inspection of vacuole images (FM-4-64). Using a program previously developed, [15] the vacuole membrane was segmented in three-dimensions and measured to give vacuole volume and surface area of individual vacuoles in individual cells. Total vacuole sizes were calculated by summing the sizes of all individual vacuoles in the cell, and average vacuole sizes were calculated by averaging the sizes of all individual vacuoles in the cell. For experiments involving nucleus position, nucleus centroids were manually annotated based on either a negative staining pattern from FM-4-64 which often highlights nucleus position [figure 1(G), WT + TBZ cells] or a GFP-label for nuclear pore complex proteins [figure 1(D), rsp1-1].

In order to locate vacuole positions within the cell, the cell major axis was first defined by drawing a line between two cell tips manually identified in the brightfield z-stack. The angle of the major axis with respect to the image axes was recorded and used to apply a rotational transformation to both the major axis and the center-point coordinates of all the vacuoles in the cell. The major axis was divided into equal-length sections, and vacuoles were binned into these sections to be counted. Typically, eight sections were defined so that section 1 = new tip, section 4 and 5 = cell midpoint, and section 8 = old tip. Additionally, the number of vacuoles between the nucleus and the new and old tips was calculated.

To map the available cytoplasmic space in each of these sections, we assumed that the tips can be accurately represented as hemispheres, and the regions between where they stop curving as roughly cylindrical [16]. Radii for new and old tips were measured independently as they are not necessarily the same. Previous research has indicated that the nuclear-cell size ratio is consistent with a value of 8%, [14] and so this was modeled as a spherical object with the appropriate volume typically positioned at the cell midpoint between sections 4 and 5. This model was used to calculate the percentage of cytoplasmic volume in each of the eight sections for comparison with vacuole distributions.

2.5. Machine learning for vacuole detection and cell segmentation

Image processing to measure cell and organelle size and distribution represents a limiting step in data analysis for large-scale experiments. We used advance image processing and machine learning tools to automate two major tasks that in current basic analysis require manual annotation (figure 2). The first task is to predict vacuole centroids from 3D fluorescence image stacks, and these centroids are used with the algorithm in reference [15] to segment
vacuoles and measure their size. The second task is to segment fission yeast cells by outlining their contours. We approach these tasks by training machine learning algorithms with manually annotated images from the *nij2Δ* set of images. Specifically, we use a single neural architecture, a U-net convolutional neural network (CNN), to perform both tasks [17].

Our pipeline functions as follows. The vacuole-centroid task uses a neural network that locates vacuoles in the 3D image stack taken of FM-4-64 (vacuole membrane). However, the neural system does not operate in 3D image space. Instead, it first predicts the centers of vacuole cross-sections in individual 2D image slices, providing an output matrix for each image that shows the confidence score of the neural detection. Outputs from all slices are combined, so that the predicted centers are clustered in 3D space to show the extent of a vacuole in a consecutive set of z-slices. The centroids of these clustered predictions are used to predict their locations in the whole image stack. The predicted center locations serve as input for the shooting-ray algorithm described in reference [15] that reconstructs the three-dimensional surface of the vacuoles and provides measurements like vacuole volume and surface area.

The second task is to predict the cellular outline of the *pombe* cells, which allows for assigning vacuoles to cells and measuring their cellular distribution. As shown in figure 2 another neural net is trained using three projections images (maximum, mean, and standard deviation) that are derived from 3D brightfield image stacks taken of fission yeast cells.

Though the proposed workflow involves two recognition tasks, namely, detection and segmentation, it is made efficient with the use of a single U-net style autoencoder–decoder architecture that performs both tasks. Although the architecture is same, the input–output pairs, along with the loss functions, are different in the two machine learning tasks. Use of a single backend architecture makes our implementation and the source code relatively simple to manage. Such a design choice keeps the overall design of our workflow compact yet effective. We report extensive details and results of the algorithms, as well as comparisons with other state of the art methods in an appendix in the supplementary information (https://stacks.iop.org/PB/17/065004/mmedia).

2.6. Output and validation from computer vision tasks

Figure 3(A) illustrates the input and output of our fully convolutional regression network for vacuole centroid detection. The output of the machine learning algorithm was compared with the ground truth from manual annotation to produce precision-recall scores calculated according to supplementary equations (S1) and (S2), both of which were found to be ∼0.8. Figure 3(B) illustrates the results of our machine learning algorithm developed for segmenting cells. It is seen that the supervised U-net stands out from two other unsupervised methods in its power to accurately predict cell contours, especially when the cells are closely spaced (figure S12). Extensive additional information is included in an appendix in the supplemental information.

3. Results

3.1. Scaling relationships between vacuole and cell size

Previous studies of the nucleus show extremely consistent correlations between organelle and cell size [14]. Therefore, our first experiments tested whether total vacuole size (the sum of all individual vacuole sizes in a cell) correlates with an increase in cell size
Figure 3. Input and output for machine learning algorithms. (A) Vacuole centroid localization occurs in 3D. The top row shows three consecutive image slices taken from a z-stack of FM-4-64 labeled vacuoles used as input. The bottom row shows the predicted scoremap of the machine learning algorithm. Insets show a magnification of the vacuole in the dotted yellow circle. The predicted scoremap shows a faint signal when the vacuole is not fully visible (left and right), compared to a strong signal when the vacuole is at its equatorial plan (center). (B) The left column shows input images for cell segmentation derived from brightfield z-stacks. The middle column shows manually annotated binary masks of cell shape. The right column shows output from the machine learning algorithm.

As seen in figures 4(A) and (B), total vacuole volume and total vacuole surface area both show a positive correlation with cell length, regardless of strain ($p < 0.05$, ANOVA). This indicates that as cells grow, the total vacuole volume and total vacuole surface area also increase, and this result tracks with similar findings in budding yeast [6, 7].

To determine how robust vacuole-to-cell size scaling is, we asked whether changes in cell size significantly alters the observed trends. To do so, we characterized vacuoles in mutant strains that affect the ability of the cell to complete its growth and cytokinesis. cdc25 is a cell-cycle activator, and $cdc25^{-}$-mutants result in prolonged cell division cycles and longer cell phenotypes [14]. Unfortunately, attempts to measure vacuole size in $cdc25^{-}$-mutants were unsuccessful as individual vacuolar diameters approached the resolution limits of conventional optics (figure 1(B)).

We had better success measuring vacuole size in wee1-ts strains (figure 1(C)). Wee1 encodes for an inhibitor of mitotic cyclins, the cellular signal for the initiation of mitosis. Wee1-ts mutant cells enter into mitosis before cells have reached wild-type lengths, [18] and this change results in cells that on average measure half the wild-type length [table 1, figure S1(A)] [14, 18]. Even at shorter cell lengths, the total amount of vacuolar volume and surface area show a positively increasing trend when plotted against
increasing cell length (figures 4(A) and (B)). Furthermore, this trend is fully consistent with the wild-type trends \((p = 0.10, 0.89, \text{comparison of regressions, respectively})\).

Other mutants characterized in this study \([\text{rsp}1-1, \text{rgo}4\Delta, \text{pom}1\Delta, \text{ny}2\Delta]\), figures 1(D)–(F)] were not expected or observed to have drastic cell size changes (figure 4, table 1). For each of these strains, longer cells also tend to contain vacuoles with greater total volume \((p < 0.05, \text{ANOVA})\).

We wanted to determine whether vacuole growth is due to increase in the number of vacuoles through fission and new biosynthesis, and/or if it is due to increase in the average size of the individual vacuoles within a cell. In all strains characterized, larger cells show greater numbers of vacuoles \([\text{figure 4(C)}, p < 0.01, \text{ANOVA}]\), however the results for average size of individual vacuoles is less consistent. In contrast, most strains, including wild-type, do not exhibit a significant increase in average vacuole volume with cell size \([\text{figure 4(D)}, p > 0.05, \text{ANOVA}]\). The only observed exception is in \text{pom}1\Delta \([\text{figure 4(D)}, p < 0.05, \text{ANOVA}]\). Together, this evidence indicates that increase in vacuole number plays a consistent role in establishing vacuole size scaling, which likely requires either \textit{de novo} synthesis of new vacuoles, or templated synthesis of vacuoles through fission.

We were particularly interested in how cell size changes in \text{wee}1-ts might impact the growth of vacuoles. Despite their shorter average length, \text{wee}1-ts cells have a similar average number of vacuoles per cell as compared to wild-type. When comparing cells of the same length, the number of vacuoles in \text{wee}1-ts cells is nearly double that in wild-type \((p < 0.05, \text{student’s t-test})\) and in surface area \((p < 0.05, \text{student’s t-test})\), and the average volume of vacuoles in \text{wee}1-ts does not show a significant correlation to cell size \([\text{figure 4(D)}, p > 0.05, \text{ANOVA}]\). These observations indicate that the growth of \text{wee}1-ts vacuoles also occurs primarily through increasing the number of vacuoles.

3.2. Vacuole distribution with respect to the nucleus

Organelle positioning and placement is key for a variety of cellular functions, in particular for inheritance by daughter cells during cell division \([12, 13, 19]\). For fission yeast—with a symmetric division pattern and a relatively large number of vacuoles—random placement may be sufficient to ensure daughter cells receive a proportionate amount of the organelle \([13]\). In this case, we reasoned that the distribution of vacuoles would correlate with available space.
Figure 5. Vacuole distribution along the cell axis. (A) Cells were divided into eight equally spaced sections from the new tip (section 1) to the old tip (section 8). The diagram is not drawn to scale to emphasize the difference between the new and old tips. The percent of overall available cytoplasmic volume in each section was calculated according to a geometric cell model assuming the nucleus is located at the cell center. (B) The distributions of vacuoles in wild-type (total n = 198, light blue) and nvj2Δ (total n = 131, red) along the eight sections are compared to the available cytoplasmic space (dashed black bars). Disproportionately more vacuoles are preferentially located towards the nucleus than expected based on cytoplasmic volume.

Using a subset of 31 wild-type cells (selected randomly from the full 198-cell dataset), we constructed a simple geometric model to estimate the relative amount of cytoplasmic volume in eight sections of the cell, which are equally spaced along the long axis (figure 5(A)). The amount of cytoplasmic volume differs slightly between sections mirrored across the nucleus (ex. sections 1 and 8, 2 and 7, 3 and 6, or 4 and 5). These differences are due to the slight asymmetry in fission yeast cells where new tips are generally thicker than old tips. Furthermore, we exclude the volume occupied by the nucleus, which in wild-type is consistently located at the cell midpoint between sections 4 and 5, and this correction leads to a substantial reduction in available cytoplasmic space in these sections [16].

Vacuole centers were annotated and binned within the eight cell sections. The distribution pattern in wild-type (figure 5(B)) shows that 28% of the vacuoles localize to sections 4 and 5, directly adjacent to the nucleus. 41% are found in sections 3 and 6, flanking the nucleus. 26% of vacuoles localized to sections 2 and 7. Only 5% of vacuoles localized to sections 1 and 8 at the tips. Measurements of the total vacuole volume and surface area per region yield similar distribution patterns as the number of vacuoles (figure S2). We note that these patterns show a symmetry with respect to the cell midpoint that are consistent with a previous report by Mulvihill et al on vacuole distribution [11]. To test for this symmetry, we reasoned that the distribution of vacuoles in S1–4 should be consistent with an expected distribution calculated from a mirroring of vacuoles in S5–8 (so S4 is consistent with S5, S3 with S6, etc.) Chi-squared comparison of these did not show statistical significance $\chi^2(3, n = 1517) = 5.2, p = 0.16$, table S3], supporting the idea that in wild-type, vacuole distribution is mirror-symmetric. Inspection of vacuole distributions in other strains (i.e. rsp1-1) shows this symmetry can be lost, though.

We next asked if vacuoles are distributed with respect to cytoplasmic volume. To generate a model for comparison, we calculate the expected distribution of vacuoles assuming it matches the distribution of available cytoplasmic volume. Chi-squared comparison of the measured distribution of vacuoles to the expected distribution based on cytoplasmic volume showed a statistically significant difference for wild-type cells $\chi^2(7, n = 3001) = 2804, p < 0.05$, table S1]. We therefore conclude that vacuoles are not distributed randomly with respect to available space.

We further hypothesized that vacuoles might be preferentially distributed closer to the nucleus. To test this idea, we asked how vacuole position would be impacted by mutations affecting nuclear position. The rsp1 gene encodes a protein important for the function of equatorial microtubule organizing centers
(eMTOC) help maintain the position of the nucleus at the cell midpoint prior to mitosis. The rsp1-1 loss of function mutation affects eMTOC position, resulting in a higher frequency of nuclei that are located away from the cell midpoint [20]. We considered the impact that this nuclear position phenotype might have on vacuole distribution. If the network of vacuoles is coupled to the nucleus, we would expect that regardless of nucleus position, vacuoles would maintain a similar preferential distribution closer to the nucleus.

Nuclei in wild-type cells occupy a position in the center of the cell (between section 4 and 5) with very little variance. In the rsp1-1 mutant, nuclei were located in a larger range of positions (sections 3–6), consistent with previous studies [20]. Rsp1-1 cells were binned based on which of these sections the nucleus occupied. Vacuoles were located into eight cell sections as before, revealing a dramatic shift in vacuole distribution with different nucleus positions. When compared to wild-type distributions, the percentage of vacuoles is generally less in the section containing the nucleus and greater in the sections adjacent to the nucleus (figures 5(C)–(G)). For example, when the nucleus is located in section 3 (figure 5(C)), the percentage of vacuoles is decreased in section 3 and increased in the adjacent sections 2 and 4. Similar trends are seen when nuclei are found in sections 4–6. Furthermore, regardless of strain or nucleus position, few vacuoles locate to the cell tips (sections 1 and 8). Vacuole distributions in rsp1-1 cells were compared to expected distributions based on wild-type patterns, and for all nucleus positions show a significant difference ($p < 0.05$) by chi-squared tests. (Table S1 summarizes the statistical results.) This evidence suggests there is an effect that preferentially localizes vacuoles adjacent to the nucleus.

### 3.3. Vacuole partitioning between cell halves

Even though vacuoles are not distributed throughout the cell proportionately to cytoplasmic volume, we wondered how vacuoles are partitioned between the two cell halves, as this could impact the amount of vacuole inherited by daughter cells during cell division. As others have proposed, random partitioning could on average give roughly equal amounts of vacuoles to daughter cells [13]. For individual wild-type cells, the percentage of vacuoles found in the cell halves with the new and old cell tips was calculated, and on average 51% of the total number of vacuoles are located in the half of the cell closer to the new tip (figure 6(A)). This value closely matches the proportion of cytoplasmic volume in the new tip (53%) calculated from the geometric model described in the previous section.

We further reason that a random process would result in characteristic variation in vacuole partitioning for the population. To test this idea, we created a simple simulation to generate a population of cells ($n = 198$) with the same numbers of vacuoles (ranging from 6–30 per cell) as in the wild-type data set. In the simulated populations, vacuoles were placed in the new and old tip halves of the cell with a probability matching the proportion cytoplasmic volume in the new and old tips. (This process is similar to that of creating a binomial distribution, but incorporates the heterogeneity in the number of vacuoles per cell.) Figure S3 shows representative histograms of the percentages of vacuoles on the new tip side of cells derived from the simulation compared to the histogram from wild-type data. Across 10 runs of this simulation, the standard deviation ranged from 12%–15%, which is consistent with the value measured from wild-type data of 12%, further indicating that the partitioning of vacuoles in wild-type is consistent with a random mechanism.

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**Figure 6.** Vacuole partitioning to new and old cell halves. Vacuoles were counted between the nucleus and the new and old cell tip. (A) The partitioning of vacuoles in wild-type ($n = 198$, light blue), mst2Δ ($n = 131$, light red), pom1Δ ($n = 28$, dark red), and rps4Δ ($n = 60$, purple) to the new and old tip side of the nucleus are compared to the available cytoplasmic space (dashed black bars). All strains show a nearly even distribution of vacuoles in the two cell halves, though pom1Δ shows a slight change in partitioning with more vacuoles towards the old cell tip. (B) The distributions of vacuoles in rps1-1 cells (total $n = 32$) to the new and old tip side of the nucleus are compared to the distribution in wild-type (dashed light blue bars) when the nucleus is located in sections 3–6 (labels S3–S6 in the legend).

![Figure 6](image-url)
Figure 7. Comparison of vacuole distributions between TBZ-treated cells and \( \text{rps}1-1 \). Similar to figure 5, cells were divided into eight equally spaced sections from the new tip (section 1) to the old tip (section 8). TBZ-treated wild-type cells (total \( n = 72 \)) were binned by position of the nucleus in (B) section 3, (C) section 4, (D) section 5, and (E) section 6, and the distributions of vacuoles along the eight sections are compared to \( \text{rps}1-1 \) cells (dashed bars). In \( \text{rps}1-1 \) the balance of vacuoles varies strongly with nucleus position. When the nucleus is in section 3, closer to the new tip, there are relatively fewer vacuoles further towards the new tip \([\chi^2(1, n = 80) = 32, p < 0.05]\). This balance reverses as the nucleus position changes to section 7, closer to the old tip, for which most vacuoles are found closer to the new tip \([\chi^2(1, n = 33) = 23, p < 0.05]\).

(T able S2 summarizes all statistical results for this section.) These results indicate that both vacuole distribution and partitioning are impacted by variable nuclear position.

4. Discussion

4.1. Vacuole-cell size scaling indicates possible connections to cell-cycle progression

Our results illustrate a number of ways in which vacuole morphology is regulated in fission yeast. We have found that fission yeast exhibit scaling trends between vacuolar size and cell size similar to those observed in budding yeast. Vacuole volume, surface area, and number are all correlated with cell size in every strain observed, including mutants for nuclear and growth machinery localization. Even \( \text{wee}1-\text{ts} \) mutants with smaller cell sizes were found to have similar scaling trends for total amount of vacuole volume and surface area, indicating these aspects of vacuole size may be most directly regulated.

However, comparison of vacuole number-to-cell size scaling trends (figure 4(B)) shows that \( \text{wee}1 \) cells tend to contain more vacuoles than wild-type when controlling for cell size. To explain this discrepancy, we wondered if vacuole number may be better correlated to position in the cell cycle, rather than absolute cell size. Since fission yeast divide at a relatively consistent length, we normalized cells to their maximum size in order to approximate each cell’s cell-cycle stage. After this normalization, we find that the scaling trends of vacuole number to relative cell length are more consistent between the two strains, suggesting a link between vacuole fusion/fission dynamics and cell cycle progression (figure S1(B)).

4.2. Vacuoles show non-random preferential localization around the nucleus

Our results show that vacuoles are not distributed proportionally to available cytoplasmic volume, and changes in \( \text{pom}1 \Delta \) and \( \text{rga}4\Delta \) mutants that affect the transition from unipolar to bipolar growth [21–24]. Neither strain shows a significant change from wild-type (figure 6(A)), indicating that vacuole partitioning is insensitive to changes cell growth polarity.

We next asked whether vacuole partitioning was also maintained in a roughly 50%–50% balance when nuclei are displaced in \( \text{rps}1-1 \). Nuclear position in \( \text{rps}1-1 \) can dictate the site of cytokinesis, so when nuclei are off-center, there can be asymmetric cell division [20]. We therefore calculated the partitioning of vacuoles to the new- and old-tip sides of the nucleus (figure 6(B)). In \( \text{rps}1-1 \) the balance of vacuoles varies strongly with nucleus position. When the nucleus is in section 3, closer to the new tip, there are relatively fewer vacuoles further towards the new tip \([\chi^2(1, n = 80) = 32, p < 0.05]\). This balance reverses as the nucleus position changes to section 7, closer to the old tip, for which most vacuoles are found closer to the new tip \([\chi^2(1, n = 33) = 23, p < 0.05]\). (Table S2 summarizes all statistical results for this section.) These results indicate that both vacuole distribution and partitioning are impacted by variable nuclear position.

Hypothesizing that vacuole synthesis and localization may occur preferentially at sites of cell growth, we tested whether vacuole distribution...
therefore are not randomly located in the cell. Rather, vacuoles are positioned disproportionately close to the nucleus, as evidenced by the consistent localization pattern of vacuoles around the nucleus observed in wild-type and rsp1-1 mutants. We propose the following non-exclusive models that could explain these findings:

(a) Vacuoles are molecularly attached to the nucleus. Vacuoles make contacts with numerous organelles, especially during autophagic processes [25, 26]. In budding yeast, nucleus-vacuole junction (NVJ) proteins connect the two organelle membranes [27]. We determined there is at least one homolog to NVJ2 in fission yeast, though our evidence suggests that a deletion mutant of that gene does not significantly alter vacuole distribution (figure 5(B)).

(b) Vacuoles are transported towards the nucleus. As Mulvihill et al demonstrated, vacuole position is affected by mutations affecting the actin-myosin cytoskeleton [11]. Based on observations of lysosomes in mammalian cells, [28, 29] vacuoles may also be linked to the microtubule network, and the net direction of vacuole transport may be towards the nucleus, giving rise to the observed preferential localization. This hypothesis is expanded on in the next text section 4.3.

(c) Vacuoles are excluded from the cell tips. There are consistently fewer vacuoles near the cell tips than would be expected based on available cytoplasmic volume, which may indicate a physical or other form of constraint blocking vacuoles from these locations. Such a constraint could be due to other physical occupation by other organelles.

(d) Vacuoles are formed near the nucleus. Vacuole synthesis involves maturation from biosynthetic and endocytic vesicles. A combination of proximity to ER including the nuclear envelope and transport from the plasma membrane may cause vacuoles to be formed preferentially near the nucleus.

4.3. Microtubule depolymerization impacts vacuole size and distribution in wild-type

The change in vacuole distribution with rsp1-1 suggest that the microtubule cytoskeleton plays a key role in vacuole structure in S. pombe. Microtubules have been shown to have strong impacts in the structure and distribution of endomembrane organelles in a variety of systems, [30, 31] including lysosome distribution in mammalian cells [28]. To test this possibility, we exposed wild-type cells to the microtubule depolymerizing drug thiamendazole (TBZ), and measured their vacuole sizes and distributions.

Consistent with previous studies using MT depolymerizing drugs, [11] we found that the average volume of individual vacuoles is increased in TBZ-treated cells (1.1 ± 1.1 μm³) compared to untreated (0.45 ± 0.39 μm³) (p < 0.05, student’s t-test). When we compared various scaling trends, we found that total vacuole surface area-to-cell length trends were nearly indistinguishable [figure 4(B), p = 0.94 comparison of regressions]. However, TBZ-treated cells show a nearly significant decrease in the vacuole number-to-cell size scaling trends [figure 4(C), p = 0.06 comparison of regressions] compared to untreated wild-type cells. These data strongly suggest that microtubule disruption results in existing vacuoles fusing together then expanding to a roughly spherical shape, which would explain why the total vacuole surface area remains consistent, while the number of vacuoles seems to decrease and the average volume of individual vacuoles increases.

This provides further evidence that the microtubule cytoskeleton plays a role in establishing vacuole structure, with a specific impact on the fusion and fission dynamics of vacuoles. We note that while our observations of how vacuole size and number change with microtubule disruption qualitatively agree with those of Mulvihill et al, they additionally found that microtubule disruption reduces the extent of vacuole fusion under specific conditions of osmotic shock, which indicates a complex interplay between microtubule effects and cellular stress response.

We also tested whether disrupting microtubules would also affect vacuole distribution, in particular the preferential localization to the nucleus. We found that the nucleus in TBZ-treated cells was not constrained to be at the cell mid-point, similar to rsp1-1 cells. Therefore, following the same procedure described earlier, we binned cells based on nucleus center location in sections 3–6 of the cell, and counted vacuole distribution in sections 1–8. Figure 7 shows a comparison between vacuole distributions in TBZ-treated cells and rsp1-1 mutant cells. In general, we find that compared to rsp1-1 mutant cells, vacuoles in TBZ-treated cells are reduced in abundance in the sections including and adjacent to the nucleus, and they are increased in abundance in more distal sections. For example, when then nucleus is in section 3, TBZ-treated cells contain fewer vacuoles in sections 2–4, and more in other sections that are further away from the nucleus, when compared to rsp1-1 cells.

To determine whether these changes in distribution are significant, we performed chi-squared tests comparing the vacuole distributions in TBZ-treated cells against expected distributions assuming the same relative distribution as rsp1-1 cells. For all nucleus positions, there is a significant difference (p < 0.05, Table S1 summarizes all chi-squared test results.) We do not believe this change in distribution can be solely explained due to the observed increase in individual vacuole size, as based on our geometric model, the larger vacuoles in TBZ-treated cells can still fit when located preferentially in sections adjacent to the nucleus. We conclude that the disruption of
microtubules reduces the preferential localization of vacuoles to the nucleus, providing support for Model #2 in which the microtubule cytoskeleton acts to position vacuoles in the cell, which is consistent with other studies of organelle interactions [28, 31].

Our results provide new evidence for the roles that the cytoskeleton plays in establishing organelle distribution in S. pombe. Interestingly, Mulvihill et al found disruptions to actin-myosin leads to vacuole distributions that are more concentrated towards the nucleus [11]. Combined with our observations, this leads us to propose that the microtubule and actin-myosin cytoskeletons play complementary roles in vacuole positioning in S. pombe, in which the microtubule-associated network positions vacuoles closer to the nucleus, while the actin-myosin network move vacuoles further away.

4.4. Vacuoles may be randomly partitioned between the two cell halves

We measured the number of vacuoles in the new and old tip halves of cells as a way to determine the expected amount that would be inherited by daughter cells after cell division. Vacuoles in wild-type and several other strains are split nearly evenly between the two cell halves. Interestingly, for wild-type, the standard deviation in the percentage of vacuoles on the new tip side is consistent with the expected value derived using simulations for random placement of vacuoles. Thus, vacuole distribution in the cell seems random with respect to certain aspects (partitioning between halves), but non-random in respect to others (preferential localization around the nucleus).

Furthermore, results from rsp1-1 indicate that vacuole distribution can adjust in response to changes in nuclear position, so that when the nucleus is located asymmetrically toward one cell tip, more vacuoles are found toward the opposite cell tip. This finding suggests that vacuoles move from one side of the nucleus to the other, and this property may allow vacuoles to redistribute evenly during mitosis and cytokinesis which the nucleus undergoes migration from the cell equator to the cell tips and back [32].

5. Conclusions

Our results show that vacuole size scales with cell size and as cells grow, so do the numbers of vacuoles. In wild-type, this growth occurs because both the number and average size of these vacuoles increases. In most other mutants studied, average vacuole size remains fairly constant, and cells instead adjust only the number of vacuoles. With respect to vacuole distribution in the cell, our results reject a model where vacuoles are positioned in the cell based solely on the space available. Rather, the vacuole system shows preferential localization around the nucleus, where there is less available cytoplasmic space, and this localization persists in mutant strains with mis-localized nuclei. Furthermore, upon microtubule disruption, vacuoles partially lose this localization around the nucleus, suggesting that the microtubule-associated network is an active player in this phenomenon. We propose that the effect of the microtubule-associated network on vacuole positioning balances that of actin-myosin, and this balance will be tested in future studies. Vacuoles tend to be evenly partitioned between the two cell halves, and exhibit the ability to change distribution around the nucleus if it is off-center, which may be important for proper vacuole redistribution during cell division. Future work will test this idea by observing the dynamic movements of vacuoles, as well as the functional impact of vacuole size regulation. This work will be enhanced by the described development of new tools to automate measurements of cell size and vacuole location using machine learning algorithms.

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