CHARACTERIZATION OF VACUOLE AQUAPORIN FUNCTION AND ITS IMPLICATION IN MEMBRANE FISSION

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A Thesis
in
The Department
of
Biology

Presented in Partial Fulfillment of the Requirements
for the degree of Master of Science (Biology) at
Concordia University
Montreal, Quebec, Canada

September 2013

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis prepared

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Entitled: Characterization of vacuole aquaporin function and its implication in membrane fission

and submitted in partial fulfillment of the requirements for the degree of

Master of Science (Biology)

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ABSTRACT

Characterization of vacuole aquaporin function and its implication in membrane fission

Dipti Patel

All cells and organelles must regulate volume to control their size, shape and copy number. Volume homeostasis, in turn, is dependent on osmosis facilitated by aquaporins. Despite being critical for membrane fusion and fission events that underlie organelle morphology and membrane trafficking, volume regulations in the endocytic pathway and the aquaporins responsible have not been characterized. Thus, the primary goals of my dissertation research were to better understand the role of osmosis in membrane fusion or fission and identify the aquaporin(s) responsible using the yeast vacuole as a model. First, I characterized osmosis across isolated vacuole membranes and identified the aquaporins responsible, Aqy3 and Fps1, using stopped-flow fluorometry. Fluorescence microscopy revealed that knocking out AQY3 or both aquaporins disrupted vacuole morphology, indicative of an impairment in membrane fission. Using a new cell-free vacuole fission assay, I then determined that osmosis can drive fission *in vitro* and that this process involves inactivation of Ytp7, a Rab-GTPase and key regulator of vacuole morphology. These results support a model whereby water efflux by Aqy3 permits membrane invagination while signaling the fission machinery necessary for membrane fission.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God almighty for his grace and guidance. I also want to take this opportunity to thank everyone who helped me complete my thesis research:

I cordially thank my supervisor Dr. Christopher Brett for his excellent tutoring, and teaching me how to test ideas while paying attention to experimental details. I am grateful to my co-supervisor Dr. Jean-Louis Schwartz for his guidance and collaboration, and Vincent Vachon for teaching me how to perform stopped flow fluorometry experiments at the Université de Montréal. I thank Mahmoud Karim for the preparation of the protein reagents needed for my experiments and Gabriel Lapointe for help with fluorescence microscopy. I thank Drs. Patrick Gulick, Michael Sacher, and Selvadurai Dayanandan for serving on my research committee and offering scientific guidance and advice. I thank my lab-mates Mahmoud Karim, Joel Richard, and Mark Guterman for advice pertaining to science, school and life. I thank members of the Sacher, Titorenko, and Gulick laboratories for reagents and protocols. I thank NSERC for funding my research, and GEPROM for providing me with a partial scholarship during my studies at Concordia.

I am deeply thankful to my husband and my son for their patience, love and support, without which I wouldn't have reached this present stage. I am thankful to my family and friends who helped me through all my ups and downs, and encourage me to always strive to be a better scientist.

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INTRODUCTION

Osmosis determines cell and organelle volume and morphology

Osmosis is the net movement of water across a semipermeable membrane from lower to higher concentration of solute (or osmolyte) to achieve equilibrium. As water constitutes most of an organism's mass, it is the key determinant of volume. Cells increase their volume, or swell, when they are placed in hypotonic solution or decrease their volume, or shrink, when placed in hypertonic solution due to osmosis. Hence, cellular osmolarity is a critical determinant of cell volume, and changes in osmolarity underlie morphological (size and shape) changes that permit cell differentiation, migration, and division; events that underlie diverse physiology (Monzani et al., 2009).

Like cells, organelles also change volume in response to osmosis to control their size, shape and number required for function (Sugiya et al., 2008). One the best-characterized examples is the yeast vacuole, the terminal compartment of the endocytic pathway whose primary function is protein and lipid degradation (similar to the metazoan lysosome), but it also acts as a store for cellular water to resist dehydration (Klionsky et al., 1990)(Hicke et al, 1999). Normally *S. cerevisiae* has 2 or 3 spherical vacuoles, however in response to hypertonic stress, vacuoles shrink and fragment within minutes, resulting in 5-10 smaller vacuoles (Denis and Cyert, 2002). In response to hypotonic stress, vacuoles within the cell rapidly swell and fuse to form a single, large vacuole (Bone et al., 1998). Despite changes in size and number, vacuoles retain their spherical shape, leading to a model based on

allometric scaling, whereby the cell maintains vacuole size, shape and number by tight regulation of lumenal volume – determined by osmosis – with respect to membrane surface area –determined by fission and fusion events (Brett and Merz, 2008). According to this model (see Figure 1A), membrane surface must decrease when vacuoles fuse to retain their spherical shape, which is achieved by the formation of an internalized membrane fragment as a byproduct of fusion (Wang et al., 2002). Conversely, volume must decrease to accommodate membrane collapse required for fission. Isolated vacuoles are capable of fusion and fission *in vitro*, and isolated vacuoles fuse in response to hypotonic shock (Brett and Merz, 2008) (Michaillat et al., 2012), suggesting that the mechanisms that control vacuole size, shape do not require complex cellular signaling, as originally proposed (Schüller et al., 1994)(Mager and Varela, 1993). The mechanisms that control homotypic vacuole fusion and, to a lesser degree, fission are well characterized (Wickner et al., 2002) (Michaillat et al., 2012), and a component of this machinery, the Rab-GTPase Ypt7, is crucial for regulating homotypic vacuole fusion in response to osmotic stress (Brett and Merz, 2008). Similarly, inactivation of Ypt7 is implicated in vacuole fission in response to hypertonic stress, but whether it directly contributes to this process is not known. Indeed, volume regulation at the vacuole is not understood in detail: the mechanism(s) that mediate water transport across the vacuole membrane are unknown, and how these mechanisms may contribute to vacuole fission is not defined. Because water channel proteins (called aquaporins) regulate osmosis at the plasma membrane to control cell volume and morphology, we hypothesized that an aquaporin may be present on the vacuole where it likely

contributes to observed changes in vacuole morphology in response to osmotic stress.

Aquaporin function, phylogeny and structure

First described by Agre and colleagues (Saboori et al., 1988), aquaporins are protein channels that translocate water across membranes. Aquaporin function is best characterized on the surface of cells where, in mammals for example, they regulate cell volume to allow erythrocytes to squeeze into capillaries with narrow diameters to facilitate gas exchange, or to change the volume of lens fiber cells to focus light on the retina (Borgnia and Nielsen, 1999). Aquaporins on the apical membranes of epithelial cells lining the collecting duct of the nephron are critical for water reabsorption required for whole body volume homeostasis (Knepper et al., 1996). Although most aquaporin genes in every eukaryotic genome are expressed on the surface of cells, the remaining paralogs (e.g. human AQP8, AQP11, AQP12) have been observed on organelles, including mitochondrial membrane, endosomes, lysosomes and ER (Mizutani et al., 2006)(Amiry-Moghaddam et al., 2005)(Marchissio et al., 2012) (Nozaki et al., 2008). Unfortunately, these intracellular aquaporins are poorly characterized although it is hypothesized that they also play a critical role in volume regulation underlying changes in organelle morphology.

Besides cellular location, members of aquaporin family can be further subdivided into two categories according to substrate specificity: Classical aquaporins, which conduct water exclusively, and aquaglyceroporins, which

conduct water as well as other uncharged solutes such as glycerol (Borgnia and Nielsen, 1999). Whereas humans have 13 aquaporin genes (Verkman, 2005) most laboratory strains of *Saccharomyces cerevisiae* (*e.g*. S288c) have three (see Figure 1B): AQY1, an aquaporin exclusively found on the plasma membrane where it contributes to cellular volume regulation (Carbrey et al., 2001); FPS1, an intracellular aquaglyceroporin (Luyten et al., 1995); and YFL054C (which we have named AQY3), an uncharacterized gene that shows sequence similarity (29%) to FPS1 in yeast, but greater similarity (37%) to a classical human aquaporin AQP7, thought to reside on endocytic organelles.

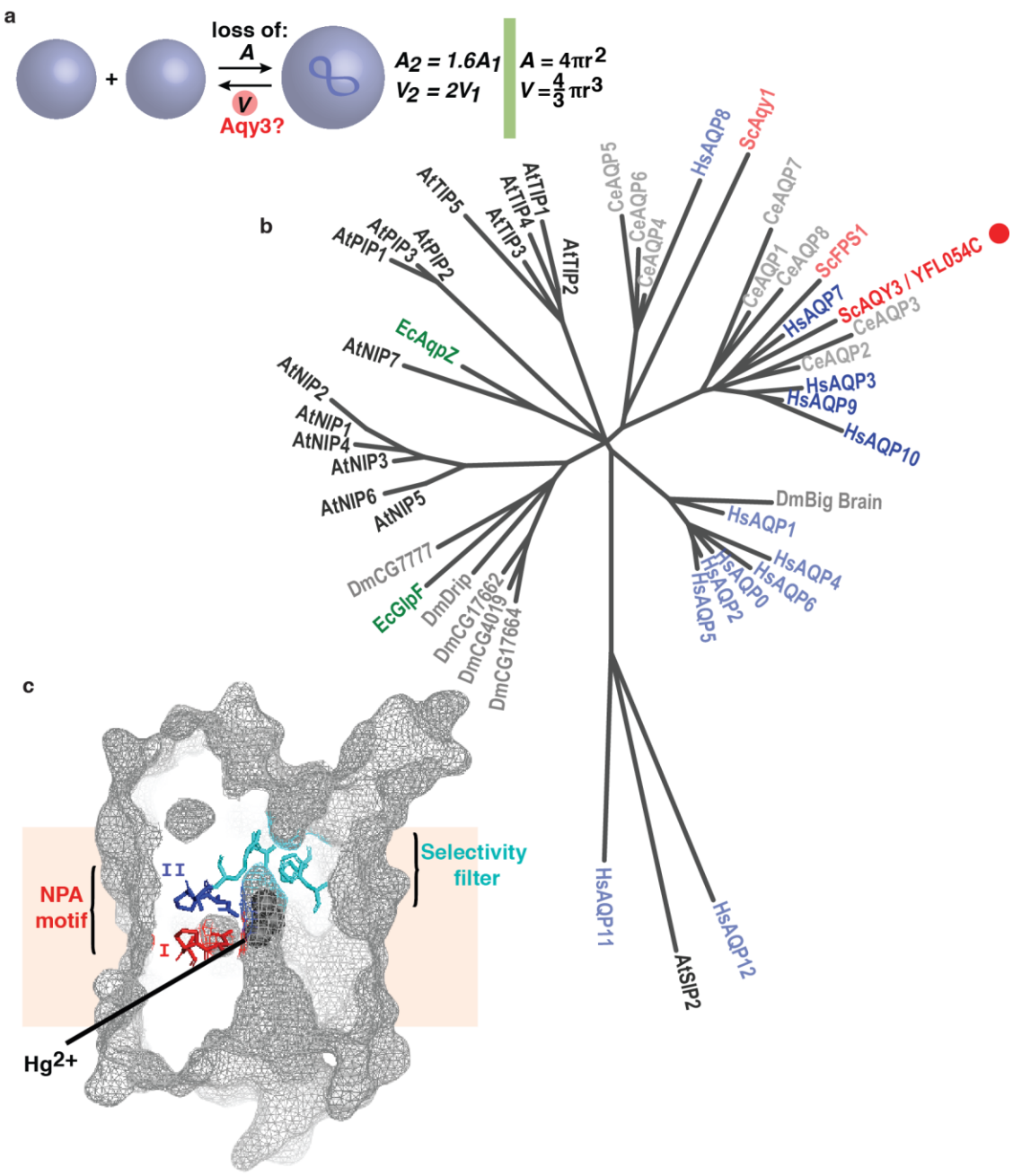
Classic aquaporins function as homo- or hetero-tetramers (Fetter et al., 2004). Each has two tandem repeats that fold into a six transmembrane-helix structure with two opposing hydrophobic loops that each contain an NPA (Asn-Pro-Ala) sequence motif (Figure 1C; (Agre et al., 1993)). These NPA motifs meet near the most narrow region of the central hourglass-shaped channel to form one of two selectivity filters (Walz et al., 1997) (Murata et al., 2000). At this site, pore diameter (3Å) and the lining residues restrict size and charge, respectively, to create a narrow, electrostatic filter that only allows permeation of water molecules (Murata et al., 2000; Sui et al., 2001; De Groot and Grubmüller, 2005). The classic water channel-blocker Hg^{2+} occludes this region of the pore (Savage and Stroud, 2007). Importantly, the NPA motifs and other critical residues necessary for water transports are conserved in AQY3, suggesting that it is a bone fide aquaporin that is sensitive to blockade by Hg^{2+} (Figure 1C). Thus, it is possible that AQY3 or FPS1

contribute to vacuole volume regulation and that inhibition of aquaporin function by Hg^{2+} may reveal how they contribute to morphology.

All things considered, the central goals of my thesis research were to identify the aquaporin(s) that mediate osmosis across the vacuole membrane, and to demonstrate that this aquaporin alters GTPase signaling to drive vacuole membrane fission in response to hypertonic stress. To achieve these goals, I first characterized the osmolytes important for the osmotic gradient across the vacuole membrane using stopped-flow spectrofluorometry. I then showed that the aquaporins Fps1 and Aqy3 are required for osmosis at the vacuole, and they are important for vacuole morphology within yeast cells. Finally, using a new cell-free assay to measure vacuole fission, I demonstrate that hypertonic stress induces vacuole fission *in vitro*, eliminating the need for complex cell signaling, and show that osmosis inactivates Rab-GTPases to mediate this process.

Figure 1. ***Aquaporin structure, phylogeny and function***

(a) Cartoon illustrating that vacuole surface area (SA) and volume must change to accommodate its spherical shape during fusion and fission events, respectively. Equations used to calculate the area and volume of a sphere are shown for reference. I hypothesize that AQY3 may contribute to the decrease in lumenal volume required for fission. (b) Phylogenetic tree showing relationships between aquaporin genes found in Ce, *Caenorhabditis elegans* (light grey); At, *Arabidopsis thaliana* (black); Dm, Drosophila *melanogaster* (grey); Ec, *Escherichia coli* (green); Sc, *Saccharomyces cerevisiae* (red); and Hs, *Homo sapiens* (blue). (c) Crystal structure of mercury bound within AqpZ from *E. coli,* adapted from (Savage and Stroud, 2007). The protein surface is highlighted with wire frame (grey), and components of the central selectivity filter are shown: NPA I (red), NPA II (blue), four amino acids in selectivity filter (dark cyan). Mercury atoms (dark grey spheres) block the channel by occluding the part of the pore near these regions. For reference, the membrane (orange line, width ~20 nm) is shown. A sequence alignment between yeast, human and *E. coli* aquaporin genes is shown below. NPA motif sequences are highlighted.



			I		II
ScAQY3	399	ISGGHI	NPA	VT……VGCLMALLDDSNA---PPGNGMTALIIGFLVAAIGMALGYQTSFTI	NPA	RD 551
ScFPS1	345	ISGAHL	NPS	IT……QAGTFALTDP----YTCLSSDVFPLMMFILIFIINASMAYQTGTAM	NLA	RD 519
ScAQY1	111	VSGGAL	NPA	MS……CLTVLMTAVE-----KRETNFMAALPIGISLFIAHVALTAYTGTGV	NPA	RS 234
HsAQP1	65	ISGAHL	NPA	VT……VLCVLATTDR----RRRDLGGSAPLAIGLSVALGHLLAIDYTGCGI	NPA	RS 192
HsAQP3	76	VSGAHL	NPA	VT……IVCVLAIVDPYNN---PVPRGLEAFTVGLVVLVIGTSMGFNSGYAV	NPA	RD 219
HsAQP7	87	ISGAHM	NAA	VT……QLCLFAITDQENN---PALPGTEALVIGILVVIIGVSLGMNTGYAI	NPS	RD 230
HsAQP9	77	VSGGHI	NPA	VS……LIIVFAIFDSRNL---GAPRGLEPIAIGLLIIVIASSLGLNSGCAM	NPA	RD 220
EcAqpZ	56	ISGGHF	NPA	VT……LLVIHGATD------KFAPAGFAPIAIGLALTLIHLISIPVTNTSV	NPA	RS 190

MATERIALS AND METHODS

Yeast strains

All strains of *Saccharomyces cerevisiae* used in this study are shown in Table 1. Most strains were obtained from the knock-out collection of 4,828 individual Mat-α haploid *S. cerevisiae* strains, each lacking a different non-essential gene (Invitrogen, Carlsbad, CA): *pep4*Δ and *pho8*Δ were used to conduct cell-free homotypic vacuole fusion assays, whereas wild type, *aqy3Δ*, and *fps1Δ* strains were used for stopped-flow fluorometry experiments and to study vacuole morphology *in vivo* by fluorescence microscopy. SEY6210 Pho8-GFP yeast (a kind gift from Dr. Alexey Merz, University of Washington, Seattle, WA, USA) were used to examine vacuole water influx, and SEY6210 *pep4Δ* Vph1-GFP yeast (a kind gift from Dr. William Wickner, Dartmouth College, Hanover, NH, USA) were used to conduct cell-free vacuole fission assays.

The Longtine method of genomic insertion by homologous recombination was used to insert GFP and URA3 in-frame behind AQP3 in the genome, or to replace genomic copies of AQY3 or FPS1 with HIS3-MX (Longtine et al., 1998). To knock out FPS1, linear DNA fragments were fabricated using two-step PCR: First HIS3-MX was amplified from pFA6a-His3MX6 using the forward primer 5′CCAAGTACGCTCGAGGGTACATTCTAATGCATTAAAAGACCGGATCCCCGGGTTAATT AA-3′ and the reverse primer 5′- TACCGGCGGTAGTAAGCAGTATTTTTTTCTATCAGT CTATGAATTCGAGCTCGTTTAAAC -3′, and then the homologous regions flanking the FPS1 gene were extended to 80 nucleotides using the forward primer 5′-

TTGTCCCAATAAGCGTCGGTTGTTCTTCTTTATTATTTTACCAAGTACGCTCGAGGGTAC – 3’ and reverse primer 5’ – ATGCAATCATCTATGTAAATATATATATATATATATATT ATACCGGCGGTAGTAAGCAGT –3.’ The PCR product was then transformed into BY4742 *aqy3Δ* to generate the *aqy3Δfps1Δ* strain (named DP1). The same approach was used to knockout AQY3, using the forward primer 5′- CAGTATAACGCTCCTCTGATATATGATCTAGACCCAAGTACGGATCCCCGGGTTAATTAA -3’ and the reverse primer 5′- TATGTCGTCAAATAATAAGTTCGTGAAATTAATAATTA GGGAATTCGAGCTCGTTTAAAC -3′ for the first PCR, and the forward primer 5’- GTCTCCCCCTTATTCATATAAAAAGAAGCGTATAATCGCACAGTATAACGCTCCTCTGAT – 3’ and reverse primer 5’ – AAGTAAGAGATGGTAACAAAGTCACGGCTCCCGGATGTA GTATGTCGTCAAATAATAAGT– 3’ for the second extension PCR. The final PCR product was transformed into SEY 6210 Pho8-GFP to create SEY 6210 Pho8-GFP *aqy3Δ*::HIS3MX (DPY2). GFP was inserted into the genome in-frame after AQY3 using the same method, with the first forward primer 5’- CACTGGCAGCAAGAAATCT GTGCCTACTTCGTCAGGAGGACGGATCCCCGGGTTAATTAA -3’ and first reverse primer 5’- TATGTCGTCAAATAATAAGTTCGTGAAATTAATAATTAGGGAATTCGAGCT CGTTTAAAC –3’, and second forward primer 5’- ATGATGGCACTGTCTGAGATGAATC TGGTGTTAACAGCAACAGCAACACTGGCAGCAAGA -3’ and second reverse primer 5’ – AAGTAAGAGATGGTAACAAAGTCACGGCTCCCGGATGTAGTATGTCGTCAAATAATAAG T – 3’. The final PCR product was transformed into BY4742 to generate a BY4742 AQY3::GFP strain (DPY3). All genomic deletions or insertions were confirmed by PCR.

Yeast transformation was carried out in *S. cerevisiae* cells by using the lithium acetate procedure developed by Gietz et al (Gietz et al., 1992). Unless otherwise noted, yeast were grown in either YPD (1% yeast extract, 2% peptone, 2% dextrose) or in SC media (minimal medium containing 2% glucose, 0.5 % ammonium sulfate, 0.17% yeast nitrogen base without amino acids, with or without the addition of Histidine (30 μg/ml), Leucine (0.1 mg/ml), Uracil (30 μg/ml), and Lysine (0.1 mg/ml)).

Reagents

All yeast and bacteria growth media was purchased from BIOSHOP (Bioshop, Canada Inc., Burlington, ON). All other buffers and reagents (such as wortmannin) were purchased from Sigma Aldrich (Spruce street, St. Louis, USA), with the exception of Ficoll (GE Healthcare, Hyakunincho Shinjuku-ku, Tokyo, Japan); FM4-64 (Invitrogen, Carlsbad, CA, USA); ATP (Roche, Indianapolis, IN, USA) and the Bradford Assay Kit (Pierce, Merseyside Drive, Mississauga, ON). All primers were purchased from Integrated DNA technologies (Coralville, Iowa, USA) and all restriction enzymes, polymerases and ligases were purchased from New England BioLabs Inc. (County Rd, Ipswich, MA, USA). Most consumables were purchased from Fisher (Fair lawn, New Jersey, USA) or VWR (Radnor, Pennsylvania, USA). Gyp1-46 6xHis and lyticase were expressed in *E.coli*, and purified by affinity chromatography as described (Starai et al., 2007; Lo et al., 2011; Kreis et al., 2005; Shen et al., 1991). *E.coli* (BL21, DE3) strains expressing Gyp1-46 6xHis were gifts from Alexey Merz (University of Washington, Seattle, WA, USA); strains expressing

lyticase were gift from William Wickner (Dartmouth College Hanover, New Hampshire, USA). All proteins and reagents added to fusion and fission reactions were buffer exchanged into PS buffer (20mM PIPES + 200mM sorbitol), aliquoted, flash frozen in liquid nitrogen and stored at -80 ^{0}C until use. All fusion reagent stocks were prepared in PS buffer.

Phylogenetic analysis

Protein seqences for 48 aquaporin genes from worm (*Caenorhabditis elegans*), plant (*Arabidopsis thaliana*), fly (*Drosophila melanogaster*), bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*) and human (*Homo sapiens*) were obtained from NCBI. Amino acid sequence alignments were performed using ClustalW (Thompson et al., 1994), and the final phylogenic tree shown was generated using Nearest Neighbor Interchange software (Tamura et al., 2011), whereby bootstrapping (100 replicates) and maximum likelihood mapping was applied.

Yeast vacuole isolation

Yeast cell cultures were grown overnight in 1 L of YPD medium to final cell density of 1.4 - 1.8 OD_{600nm}/ml. Cells were then collected by centrifugation at 2,824 g for 10 min at 4^{o}C. The cell pellet was washed for 10 min at 30^{o}C in 50 ml wash buffer (100 µM DTT, 50 mM Tris-HCl pH 9.4), and then collected by centrifugation at 3,435 g for 5 minutes. Cells were then resuspended in 15 ml spheroplasting buffer (25 mM potassium phosphate, pH 6.8, 200 mM sorbitol, in 1:20 diluted YPD medium) and 1-

2 µg/ml zymolyase (amount is strain dependent), and incubated for 30 minutes at 30˚C. Spheroplasts were collected by centrifugation at 1,237 g for 2 min at 4°C, and then resuspended in 2 ml of ice-cold PS buffer (20 mM PIPES, 200mM sorbitol) containing 15% ficoll. To gently disrupt the plasma membrane of cells, 0.2 – 0.4 µg/ml DEAE dextran was then added (strain dependent) to the spheroplasts prior to incubation at 30°C for 3 min. The permeabilized spheroplasts were then transferred to SW41-Ti Beckmen ultracentrifuge tube, and 8%, 4% and 0% ficoll layers were added on the top to create a floatation gradient. Tubes were then centrifuged at 125,000 g for 90 minutes at 4˚C, and isolated vacuoles were collected at the 4-0 % interphase (Haas et al., 1994). Final vacuole protein concentration was determined by Bradford protein assay. This preparation of isolated vacuoles were then used to study vacuole morphology in vitro, to perform cell-free vacuole fission and fusion assays and to measure changes in organelle volume in response to osmotic stress.

Stopped flow spectrofluorometry

Changes in vacuole volume in response to hypertonic stress were recorded over time using a light scattering assay previous employed to study mechanisms responsible for volume regulation in vesicles reconstituted from epithelial cell membranes(Kirouac et al., 2006). Vacuoles isolated from wild type, *aqy3Δ* or *fps1Δ* cells were diluted to a final concentration of 1 mg /ml in PS buffer, and rapidly injected into a cuvette with equal amounts of PS buffer containing different concentrations of osmolytes (e.g. 1.8 M glucose) using a stopped flow apparatus (Hi-Tech Scientific, Salisbury, UK). During the injection, scattered light intensity at 450

nm was monitored with a photomultiplier tube located at an angle of 90° from the incident beam using a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ). Reduction in vacuole size is observed as an increase in scattered light intensity, as the smaller vacuoles tumble more rapidly within solution causing more incidents light to scatter. To block aquaporins, vacuoles were pre-incubated with increasing concentrations of $HgCl_2$ (0.01, 0.1 or 1 mM) prior to exposure to hypertonic shock. To confirm that the osmolality of buffers containing glucose and sorbitol were equal, PS buffer containing 400 mM of either osmolyte was measured with a VAPRO vapor pressure osmometer.

Fluorescence microscopy and morphometric analysis

To study vacuole morphology *in vivo,* yeast cells were grown in SC medium overnight at 30˚C and then diluted 1:10 in a 3 ml culture of SC medium containing 3 µM FM4-64, a vital dye that stains the vacuole membrane. After incubation at 30˚C for one hour, cells were washed twice in SC medium without dye and then grown for another hour in SC medium to chase FM4-64 to the vacuole. Cells were then pelleted and resuspended in 0.5 M NaCl in SC medium to induce hypertonic shock, 2% glucose in water to induce hypotonic shock, or SC medium representing isotonic conditions. 4-6 µl of each yeast cell suspension was then transferred to a glass coverslip for fluorescence imaging using a Nikon Eclipse TI-E inverted microscope equipped with a 100 x 1.40 NA oil immersion objective lens, Photometric EMCCD camera, super bright LED light source, custom filter set to image GFP and FM4-64, and NIS Element AR V4.1 software (Nikon Canada, Mississauga, ON). Digital images

were saved as tiff files using Image/J v. 1.36b (J. Rasband, NIH) and processed using Adobe Photoshop CS5 software (Adobe System, San Jose, CA, USA). Images shown are the result of adjusting brightness and contrast levels, inverting the color and applying an unsharpen filter. To quantify the effects of osmotic shock on vacuole morphology, the number of vacuoles per cell were counted under each condition, whereby data was binned into 4 groups (1, 2, 3 or ≥ 4 vacuoles per cell). An average of 80 cells were analyzed for each strain under each condition.

In vitro vacuole membrane fission assay

To quantify vacuole fission *in vitro*, 6 µg of vacuoles isolated from SEY6210 *pep4*Δ strains expressing Vph1-GFP (a membrane-bound subunit of the V-ATPase that is uniformly distributed with vacuole membranes; see Wang et al., 2002) were added to standard fission buffer (PS buffer containing 5 mM $MgCl_2$, 125 mM KoAc, 10 mM CoA, and 1mM ATP; (Michaillat et al., 2012) and incubated for 30 minutes at 27˚C to induce vacuole fission. Fission reactions (30 µl each) were then briefly centrifuged at 3,000g for 3 minutes at 4^0C to separate smaller fragmented vacuoles (present in the supernatant) from larger intact vacuoles (present in pellet). To quantify the amount of vacuole fission that occurred *in vitro*, 20 µl of supernatant was transferred to a 96-well black conical-bottom microplate. The remaining supernatant in the reaction was then removed and the pellet was resuspended with fission buffer on ice. 20 µl of the resuspended pellet was then transferred to the same 96-well microplate and the amount of GFP fluorescence in each sample was measured using a BioTek Synergy H1 multimode fluorescence microplate reader.

The ratio of GFP fluorescence in the supernatant versus the pellet is indicative of vacuole membrane fission. All fission values obtained under each condition were normalized to the amount of fission observed under isotonic conditions in the absence of KOAc (which was replaced with equimolar amounts of KCl). To examine effects of hypertonic stress on vacuole fission in vitro, increasing concentrations of either sorbitol or glucose were added to the fission buffer where indicated. To better understand the mechanisms underlying vacuole fission, increasing concentrations of either the PI3-kinase inhibitor wortmannin or purified recombinant Gyp1-46 protein were added to the fission buffer.

In vitro vacuole lysis assay

To measure vacuole water influx, 6 µg of vacuoles isolated from SEY6210 Pho8-GFP cells with or without AQY3 were incubated with lysis buffer (20 mM PIPES-KOH pH 6.8, 50 mM sorbitol, 5 mM $MgCl_2$, 125 mM KCl) at 27˚C for 3 min. Quick exposure to hypotonic shock (50 mM sorbitol) will drive excessive water influx causing vacuoles to swell and undergo lysis releasing lumenal Pho8-GFP into the reaction buffer. After the lysis reaction is complete, each 30 µl sample was then centrifuged at 5,000 g for 5 minutes at 4˚C to separate the soluble Pho8-GFP released into the reaction buffer after lysis (present in the supernatant) from intact vacuoles (present in the pellet). To quantify the amount of vacuole lysis that occurred *in vitro*, 20 µl of supernatant was transferred to a 96-well black conical-bottom microplate. The remaining supernatant was then removed from reaction and the pellet was resuspended with lysis buffer on ice. 20 µl of the resuspended

pellet was then transferred to the same 96-well microplate and the amount of GFP fluorescence in each sample was measured using a BioTek Synergy H1 multimode fluorescence microplate reader. The ratio of GFP fluorescence in the supernatant versus the pellet is indicative of vacuole lysis. As a control to measure complete lysis, vacuoles isolated from wild type cells were treated with 1% triton X-100 to dissolved membranes. Where indicated, increasing concentrations of sorbitol was added to the reaction buffer to examine the effect of osmotic shock on lysis.

Cell-free homotypic vacuole fusion assay

In vitro homotypic vacuole fusion was measured using a simple colorimetric assay based on the maturation of the lumenal alkaline phosphatase Pho8 (see (Haas et al., 1994)). In brief, 3 µg of vacuoles isolated from *pep4*Δ and *pho8*Δ cells were mixed and added to stand fusion reaction buffer (PS buffer containing 1 mM ATP, 40 mM creatine phosphate, 0.5 mg/ml creatine kinase, 125 mM KCl, 5 mM $MgCl_2$, 10 µM CoA) and then incubated for 90 minutes at 27˚C. Upon fusion, lumenal content mixing will permit immature Pho8 to be cleaved by Pep4 to activate the enzyme. Pho8 activity is then measured by the addition of 500 µl of development buffer (250mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.4 % triton X-100, 1 mM paranitrophenolphosphate) and incubation for 5 min at 30˚C. The phosphatase reaction is then stopped with 500 µl stop buffer (100 mM glycine pH 11) and absorbance at 400 nm is measured using a NanoDrop spectrophotometer. Where indicated, KCl was replaced with equimolar KOAc or increasing concentrations of sorbitol were added to the reaction buffer. Fusion values shown represent A_{400nm}

values obtained under each condition normalized to values obtained under normal fusion conditions (e.g. 200 mM sorbitol, 125 mM KCl).

Data analysis

All quantitative data was processed using Microsoft Excel v.14.0.2 software (Microsoft Cooperation, Redmond, Washington, USA), including calculation of means and S.E.M. values. Final data sets were plotted using Kaleida Graph v.4.0 software (Synergy Software, Reading, PA, USA). All figures were prepared using Adobe Illustrator CS5 software (Adobe Systems, San Jose, CA, USA). The cartoon of the crystal structure of EcAqpZ (Savage and Stroud, 2007) (Benson et al., 2009) was prepared using PyMOL software (Portland, OR, USA). The final thesis was written and assembled in Microsoft Word V14.0.2 software (Microsoft Cooperation, Redmond, Washington, USA), and references were prepared using Mendeley software (Mendeley, New York, NY, USA).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
BY4742	*MAT*α *his3*Δ *leu2*Δ *lys2 ura3*Δ	Invitrogen, CA, USA
BY4742 *pep4Δ*	*MAT*α *his3*Δ *leu2*Δ *lys2 ura3*Δ *pep4*Δ*::neo*	Invitrogen, CA, USA
BY4742 *pho8Δ*	*MAT*α *his3*Δ *leu2*Δ *lys2 ura3*Δ *pho8*Δ*::neo*	Invitrogen, CA, USA
BY4742 *fps1Δ*	*MAT*α *his3*Δ *leu2*Δ *lys2 ura3*Δ *fps1*Δ*::neo*	Invitrogen, CA, USA
BY4742 *aqy3Δ*	*MAT*α *his3*Δ *leu2*Δ *lys2 ura3*Δ *aqy3*Δ*::neo*	Invitrogen, CA, USA
SEY6210 PHO8-GFP	*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 lys2-801; GAL* PHO8-GFP (TRP1)	Angers & Merz, unpublished data
SEY6210 *pep4 Δ* Vph1-GFP	*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 suc2-*Δ*9 lys2-801 pep4::HIS3* VPH1-GFP(TRP1)	Wang *et al*., 2002
DPY1 (BY4742 *aqy3Δ fps1Δ*)	BY4742 *aqy3Δ fps1Δ*::HIS3MX	This study
DPY2	SEY6210 PHO8-GFP *aqy3Δ*::HIS3MX	This study
DPY3	BY4742 AQY3::GFP	This study

RESULTS

Fps1 and Aqy3 contribute to osmosis across vacuole membranes

Despite playing a major role in cellular osmotolerance, the mechanisms that transport water across vacuole membranes in *S. cerevisiae* have not been identified. As a first step towards finding the vacuole water transporter, I devised a method to accurately measure volume changes during osmosis across the vacuole membrane. Water conductance through water channels is very rapid (0.5 sec), and vacuoles fuse in response to swelling induced by hypotonic shock within seconds, suggesting that water flux occurs very rapidly, i.e. within a second after stress is applied. Thus to facilitate our studies, we collaborated with Drs. Vincent Vachon and Jean-Louis Schwartz at the Université de Montréal. Drs. Vachon and Schwartz have decades of experience measuring the rapid changes in membrane vesicle diameter in response to different osmolytes using stopped flow spectrofluorometry (Kirouac et al., 2006). Under hyperosmotic shock as water escaped from vacuoles, their volume decreased rapidly, as evident by sharp increase in scattered light intensity (see Figure 2a). After significant optimization of the methodology to accurately measure changes in diameter of isolated vacuoles (which are 10 fold larger than the vesicles Drs. Vachon and Schwartz typically use in their studies), I measured the effects of osmotic stress on vacuole size using different osmolytes, whereby changes in vacuole diameter reflect changes in volume in response to osmosis (see Figure 2). Experiments employing hypotonic stress were quickly abandoned because our results suggested that the isolated vacuoles were either undergoing lysis or the threshold of detection

by the apparatus was reached (data not shown). Instead, we focused on hypertonic stress and decreases in vacuole size which were observed as increases in light scattering at 450 nm by fluorometry. We avoided adding high concentrations of salts, like KCl or KOAc, that are conventionally used to trigger vacuole fragmentation within intact yeast cells, because the concentrations necessary to elicit a hyperosmotic stress (e.g. 250 mM) completely abolished membrane fission or fusion of isolated vacuoles. Instead, we chose to initially study the effects of adding the osmolyte sorbitol on vacuole size, as it was reported to initiate changes in morphology when added to isolated vacuoles (Brett and Merz, 2008). Surprisingly, addition of sorbitol to isolated vacuoles did not cause a sustained decrease in vacuole diameter (see Figure 2b). Rather, we observed a rapid decrease in size that peaked at 500 ms, followed by a recovery to a size slightly smaller than observed under isotonic conditions. This result is expected if the osmolyte is transported across the membrane, as in the case of glycerol (see Fig 2b), which is known to cross the vacuole membrane and does not change vacuole morphology. But to comprehensively assess osmosis we decided to try other osmolytes thought to be important for cytoplasmic osmolarity that may not be transported across the vacuole membrane.

It was previously reported that addition of glucose to *S. cerevisiae* causes vacuole fragmentation, unlike other yeast species (Vindeløv and Arneborg, 2002). Thus, we applied it to the isolated vacuoles and observed rapid decreases in volume that were sustained and increased in a concentration-dependent manner (see Figure 3a), suggesting that it is an effective osmolyte that does not efficiently cross

the vacuole membrane. The change in volume occurred within 1 sec consistent with the presence of fast-conducting water channels on the vacuole membrane. To initially test this hypothesis, we pretreated isolated vacuoles with $HgCl_2$, an aquaporin blocker that lodges in the channel to prevent transmembrane water flow. Treatment with increasing concentrations of $HgCl_2$ cause progressive loss of the observed change in volume induced by 900 mM glucose, and the signal was abolished in the presence of 1 mM $HgCl_2$ (see Figure 2c). This result proposed that Hg^{2+}-sensitive mechanism, likely an aquaporin, is mediating the observed rapid change in organelle volume.

Thus, I hypothesized that one or more of the 3 aquaporin paralogs in *S. cerevisiae* may mediate osmosis across the vacuole. Because Aqy1 is only found on the yeast plasma membrane (Sidoux-Walter et al., 2004), I focused on Fps1, which is present on intracellular membranes (Beese et al., 2009) and Aqy3, an uncharacterized aquaporin protein. To study their contributions to vacuole osmosis, I conducted stopped-flow fluorometry studies with vacuoles isolated from yeast strains lacking each (*fps1Δ* or *aqy3Δ*) or both (*fps1Δaqy3Δ)* aquaporins. Deletion of AQY3 or FPS1 diminishes the changes in vacuole size caused by addition of glucose (see Figure 3b & 3c respectively), and deleting both aquaporin genes may completely abolishes the effect, similar to the effect of pretreating vacuoles with 1 mM $HgCl_2$. This is best illustrated by further analysis of the peak change and the steady state change in relative vacuole size (see Figure 3d). Knocking out FPS1 has a greater effect on the peak change than deleting AQY3; however the effect of knocking out each single gene is not an additive. Together, these results proposed

that both Fps1 and Aqy3 contribute to osmosis across the vacuole membrane, although they are not functionally redundant and may function synergistically (e.g. as a heterodimer) to control organelle volume. When I have repeated this experiment with isolated vacuoles from *fps1Δaqy3Δ* strain, it was unable to acquire consistent result (data not shown).

Figure 2. ***$HgCl_2$ blocks vacuole osmosis caused by hypertonic shock***

(a) Illustration of the water efflux assay. Isolated vacuoles are rapidly exposed to hypertonic shock while their relative volume is monitored by spectrofluorometry. (b) Changes in vacuole volume recorded in response to exposure of different osmolytes as compared to isotonic conditions. Glucose was the only osmolyte tested that caused a sustained decrease in vacuole volume. (c) Vacuole shrinking upon hypertonic stress (+ 0.9 M glucose) was blocked by addition of increasing concentrations of $HgCl_2$. Means ± S.E.M. are shown for 17 – 22 independent experiments depending on condition.

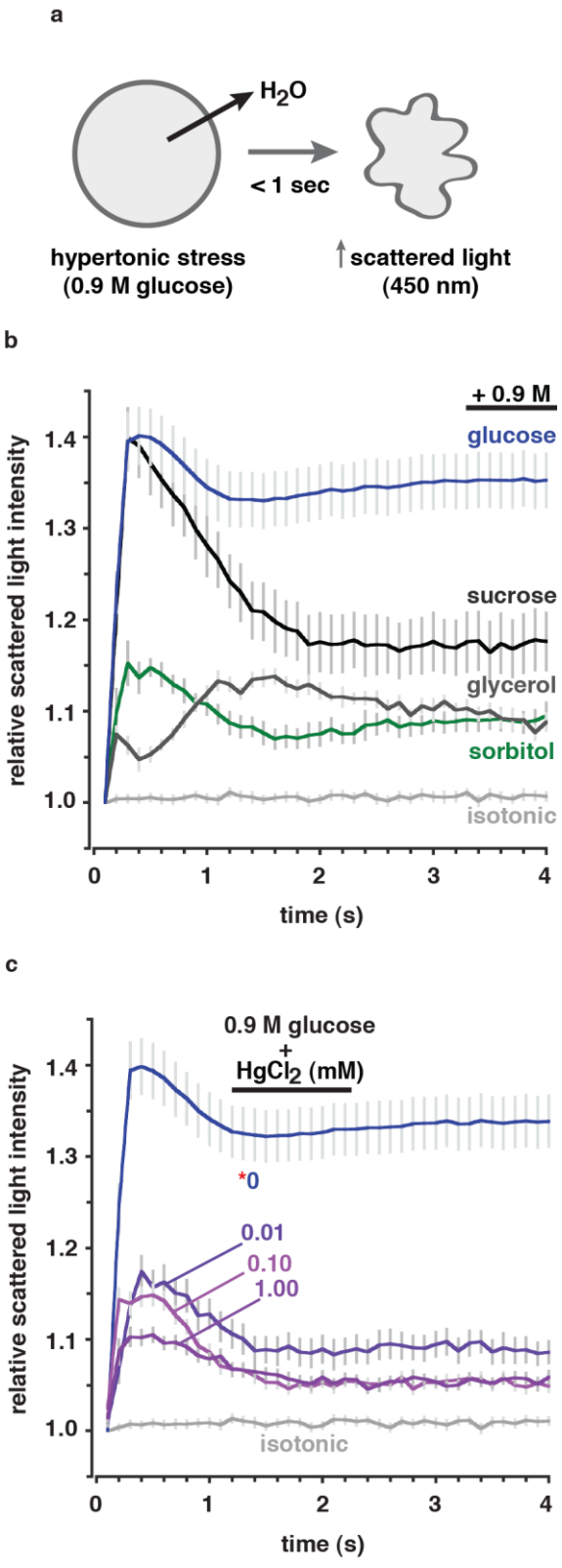
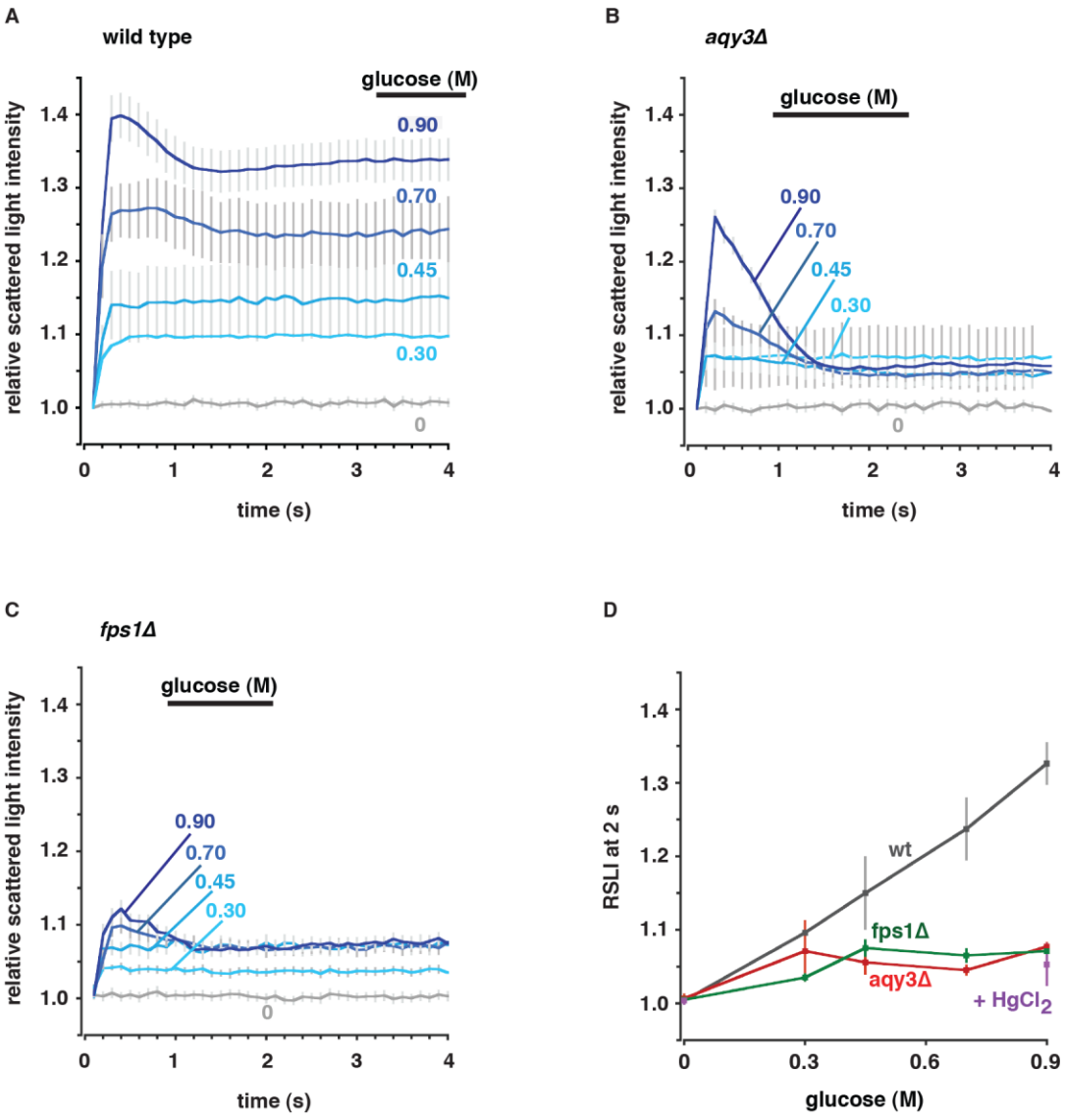

a
H_2O
< 1 sec
hypertonic stress
(0.9 M glucose)
scattered light
(450 nm)
b
+ 0.9 M
glucose
sucrose
glycerol
sorbitol
isotonic
relative scattered light intensity
1.4
1.3
1.2
1.1
1.0
0
1
2
3
4
time (s)
c
0.9 M glucose
+
$HgCl_2$ (mM)
*0
0.01
0.10
1.00
isotonic
relative scattered light intensity
1.4
1.3
1.2
1.1
1.0
0
1
2
3
4
time (s)

Figure 3. ***AQY3 and FPS1 mediate water efflux from vacuoles in response to hypertonic shock***

Isolated vacuoles from WT (a), *aqy3*Δ (b) or *fps1*Δ (c) cells were rapidly exposed to increasing concentrations of glucose while volume was measured over time using spectrofluorometry. (d) Steady state values of relative scattered light intensity (indicative of relative volume) recorded at 2 second under conditions shown in panels A-C were plotted against glucose concentration. Data was normalized to values obtained in the absence of glucose and data shown represents the mean ± S.E.M. from 4 – 67 independent experiments depending on condition.


A
wild type
glucose (M)
0.90
0.70
0.45
0.30
0
relative scattered light intensity
time (s)
B
aqy3Δ
glucose (M)
0.90
0.70
0.45
0.30
0
relative scattered light intensity
time (s)
C
fps1Δ
glucose (M)
0.90
0.70
0.45
0.30
0
relative scattered light intensity
time (s)
D
RSLI at 2 s
wt
fps1Δ
aqy3Δ
+ HgCl2
glucose (M)

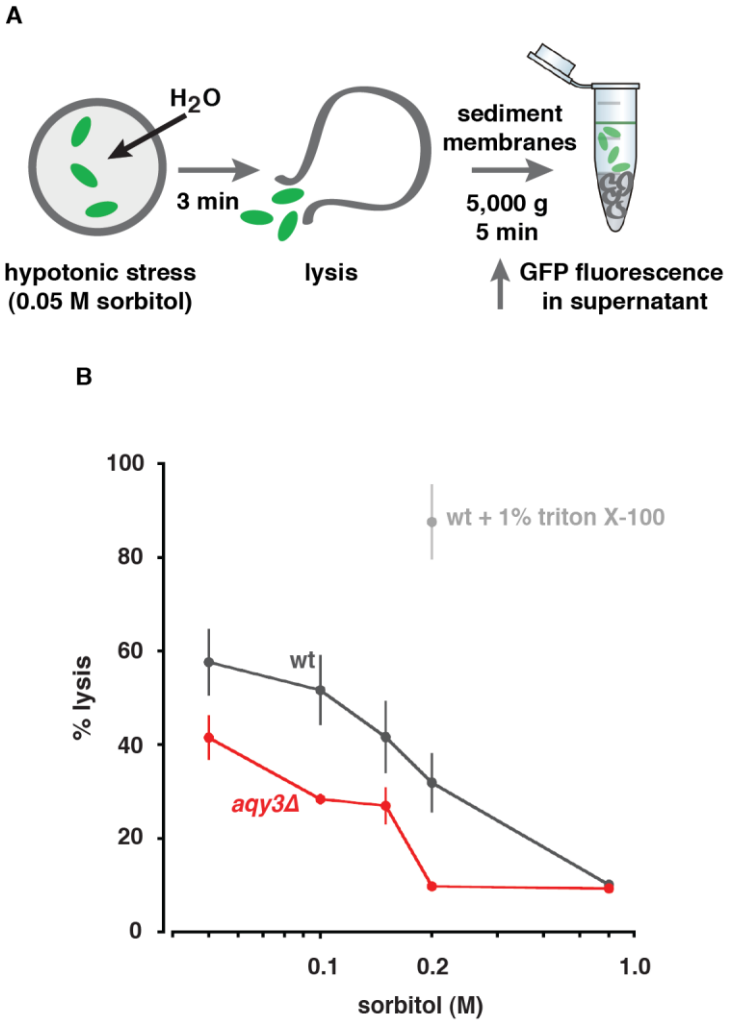
Aqy3 facilitates vacuole lysis in the absence of membrane fusion

Using stopped-flow fluorometry, we were able to demonstrate that Fps1 and Aqy3 contribute to water *efflux* from the vacuole lumen in response to hypertonic stress. However, we were not able to examine water *influx* using this method. Thus, we designed a second assay to measure osmosis across the vacuole membrane based on the following reasoning: In response to hypotonic stress, water floods into vacuoles causing them to rapidly swell and fuse (Brett and Merz, 2008). Fusion effectively increases the total relative lumenal of the vacuole compartment within the cell to accommodate the incoming water to prevent rupture. Thus, when the membrane fusion machinery is impaired, vacuoles swell and rupture (Starai et al., 2007). Under these conditions, the *extent of rupture* directly correlates with the extent of *water influx*. To measure rupture, we isolated vacuoles from cells expressing a GFP-tagged variant of Pho8, an alkaline phosphatase that is exclusively found in the vacuole lumen, subjected them to hypotonic stress in the absence of ATP (which is required for homotypic vacuole fusion *in vitro*), sedimented intact organelles & membranes and measured the amount of Pho8-GFP that escaped from lumen that was found in the supernatant (Figure 4a). Initially, I used this assay to demonstrate that progressively stronger hypotonic shocks cause a gradual increase in membrane rupture of vacuoles isolated from wild type cells (Figure 4b). Rupture is blocked by the addition ATP, that promotes membrane fusion, but this effect is reversed when vacuoles are pretreated with hypertonic stress (e.g. addition of 1 M sorbitol), which stimulates water efflux and does not cause vacuole rupture.

Next, I determined if the aquaporin Aqy3 contributes to water influx that causes vacuole membrane rupture in response to hypotonic stress; a similar assay used to first identify aquaporins by Peter Agre and colleagues (Preston et al., 1992). Deleting AQY3 reduced rupture of isolated vacuoles in response to hypotonic shock (Figure 4b). Thus, we conclude that Aqy3 facilitates bidirectional transmembrane water flux across vacuole membranes.

Figure 4. ***Aqy3 mediates vacuole water influx into vacuoles in response to hypotonic shock***

(a) Illustration of the method used to measure vacuole water influx. Isolated vacuoles were incubated at 27^0C for 3 min in 30 µl PS buffer with a range of sorbitol concentrations between 50 and 600 mM. Excessive water influx at low sorbitol concentrations causes lysis, evident by escape of lumenal GFP isolated in the supernatant after sedimentation of intact vacuoles. (b) Percent vacuole lysis values are shown for vacuoles isolated from wild type or *aqy3Δ* cells treated with reaction buffer with decreasing concentrations of sorbitol. Lysis of WT vacuoles treated with 1% triton X-100 (light grey) to disrupt membranes is shown as a positive control. Means ± S.E.M. values are shown from 4 independent experiments.


A
H_2O
3 min
sediment
membranes
5,000 g
5 min
hypotonic stress
(0.05 M sorbitol)
lysis
GFP fluorescence
in supernatant
B
100
80
60
40
20
0
% lysis
wt + 1% triton X-100
wt
aqy3Δ
0.1
0.2
1.0
sorbitol (M)

Fps1 and Aqy3 may localize to vacuole membranes

With the knowledge that deleting AQP3 gene diminishes water movement across isolated vacuoles, we hypothesized that it must be present on vacuole membrane to perform this function. In a previous study, Fps1 tagged with tdTomato at the C-terminus was localized to the vacuole as well as plasma membrane (Mollapour and Piper, 2007)(Beese et al., 2009) confirming its presence on the vacuole membrane. However, the cellular location of Aqy3 is unknown. Thus, I knocked-in GFP behind AQY3 in the yeast genome but acquire clear fluorescence micrographs because it seems that endogenous expression levels of AQY3 are too low for detection (data not shown). Thus, although our functional data shown in Figure 3 and 4 strongly suggests the presence of Aqy3 on vacuole membranes, additional experiments are necessary to confirm its presence on the vacuole.

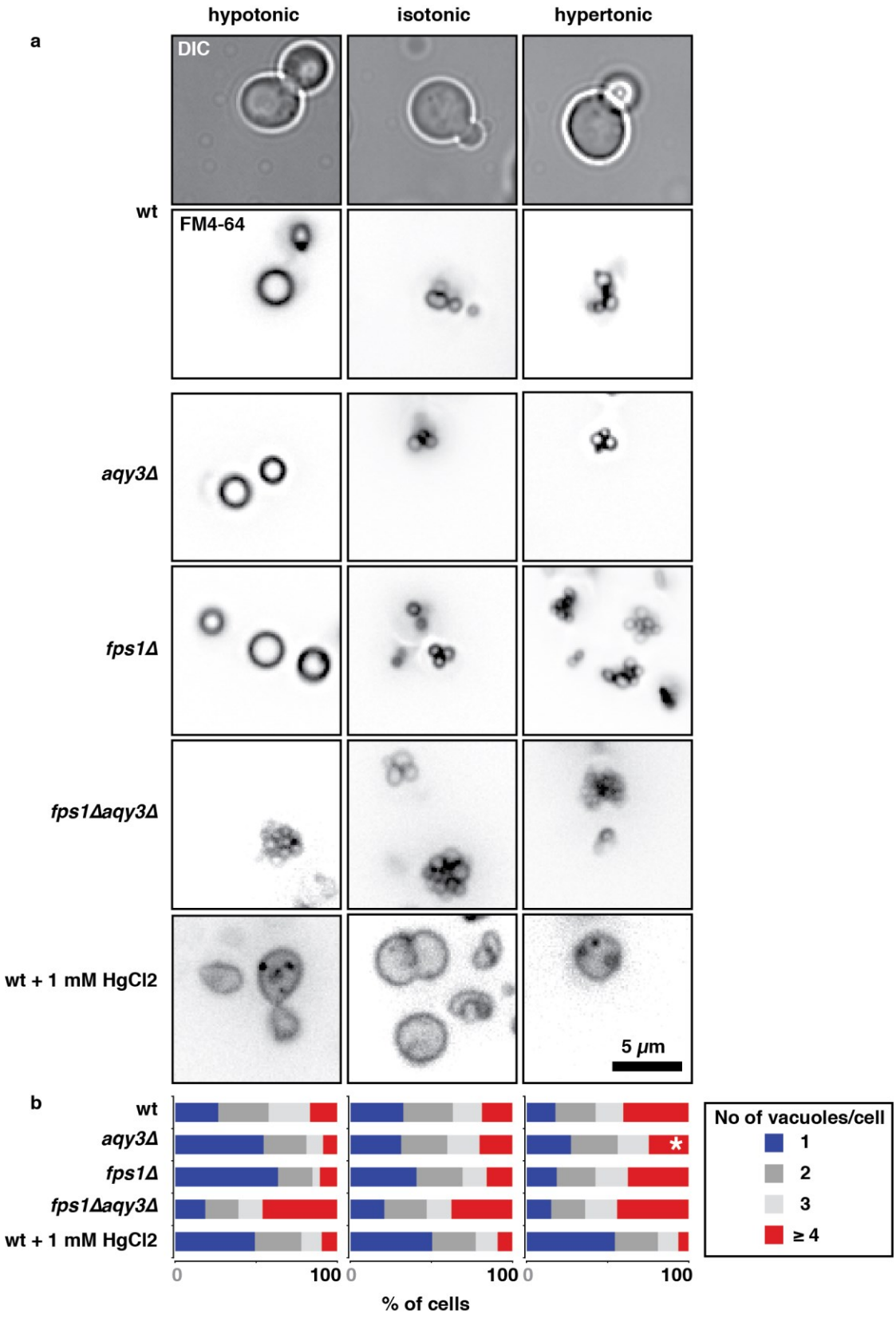
Fps1 and Aqy3 contribute to vacuole morphology

Given that Fps1 and Aqy3 contribute to osmosis across the vacuole membrane, we hypothesized that these aquaporins also contribute to vacuole morphology, as osmotic stress is known to drive membrane fusion and fission events (Weisman, 2003)(LaGrassa and Ungermann, 2005). Specifically, exposing yeast cells to a hypotonic stress causes water influx into the vacuole, and homotypic membrane fusion within seconds, whereas hypertonic stress causes water efflux and vacuole fragmentation within minutes (Brett and Merz, 2008). To determine if Fps1 or Aqy3 contribute to these observed changes in vacuole morphology, I stained vacuoles with the vital dye FM4-64 (to visualized vacuole membrane) and imaged

them using epifluorescence microscopy (see Figure 5). Yeast cells devoid of FPS1, AQY3 or both aquaporin genes were all tested. Deletion of both aquaporins causes a severe defect in vacuole morphology – constitutive fragmentation – that also abolished the response to osmotic shock, suggesting that vacuole biogenesis may be impaired. Although no significant effects were observed when only FPS1 was deleted, vacuoles within *aqy3Δ* cells failed to fragment in response to hyperosmotic shock (see Figure 5a). Together these results suggest that Fps1 and Aqy3 are important for maintaining vacuole morphology, and that Aqy3 may have a specific function in vacuole fission in response to hypertonic stress.

Figure 5. ***Knocking out AQY3 or FPS1 affects vacuole morphology in response to osmotic shock***

(a) Wild type, *aqy3*Δ, *fps1*Δ, or *aqy3*Δ*fps1*Δ cells or wild type cells treated with 1 mM $HgCl_2$ were stained with FM4-64 and imaged after 30 minutes of treatment with either water or 2 % glucose to induce hypotonic shock, SC medium containing 0.4 M NaCl to induce hypertonic shock, or SC medium, representing normal isotonic conditions. Representative fluorescence micrographs for each condition and strain are shown. DIC images of WT cells are also shown for reference. (b) The number of vacuoles per cell was quantified using microscopic data similar to that shown in (a). Percentage of cells with either 1 (blue), 2 (light grey), 3 (grey), ≥4 (red) vacuoles were then calculated and the means from four independent experiments (> 100 cells) were plotted for each strain under each condition.


hypotonic
isotonic
hypertonic
a
DIC
wt
FM4-64
aqy3Δ
fps1Δ
fps1Δaqy3Δ
wt + 1 mM HgCl2
5 μm
b
wt
aqy3Δ
fps1Δ
fps1Δaqy3Δ
wt + 1 mM HgCl2
*
0
100
% of cells
No of vacuoles/cell
1
2
3
≥ 4

Water efflux and Rab-GTPase inactivation facilitates vacuole membrane fission in response to hypertonic shock

Studies by Mayer and colleagues have used fluorescence microscopy to demonstrate that isolated vacuoles undergo membrane fission *in vitro* (Michaillat et al., 2012). But this assay has many shortcomings, the greatest being that it is qualitative. Thus, I designed and optimized a new cell-free assay to quantify vacuole membrane fission: Vacuoles isolated from yeast cells expressing GFP-tagged Vph1 (a component of the vacuolar-type H^+ ATPase that uniformly decorates vacuole membranes) were treated with ATP and 125 mM potassium acetate (KOAc) to stimulate membrane fission (Michaillat et al., 2012). After fission reactions were complete, smaller vacuole fragments were separated from larger vacuoles by differential centrifugation and the amount of Vph1-GFP found in the supernatant containing the fragments was quantified by fluorometry (see Figure 6a). A series of optimization studies revealed that centrifugation at 3,000 g for 3 minutes was best at separating fragments from intact vacuoles (data not shown), and that maximum fragmentation occurred at 30 minutes, consistent with published findings both *in vitro* and *in vivo* (Zieger and Mayer, 2012). We used fluorescence microscopy to visualize vacuoles contained in each fraction (Figure 6b) and confirmed that the fragments in the supernatant were smaller and efficiently separated from the larger vacuoles in the pellet. Furthermore, we estimated vacuole size by quasi-elastic light scattering and confirmed that the mean diameter (± S.E.M.) of the fragments was 452 ± 4 nm whereas the mean diameter of the vacuoles in the pellet was 2.15 ± 0.05 μm, similar to the diameters observed within intact yeast cells (Michaillat et al.,

2012). Assuming all vacuoles are spherical in shape (as observed by many independent research groups; (Wiemken et al., 1970)(Petekçakar et al., 2000)(Zieger and Mayer, 2012)), we estimate that one vacuole gives rise to 17 fragments upon hypertonic stress, consistent with previous reports (Michaillat et al., 2012). In addition to quantifying vacuole fission stimulated by KOAc, I also show that KOAc has the opposite effect on homotypic vacuole fusion (see Figure 6d). Like KOAc, I find that hypertonic stress, either by adding the osmolytes sorbitol (Brett and Merz, 2008) or glucose, also induces vacuole fragmentation *in vitro* (see Figure 6c & 6e). How KOAc stimulates fission is not entirely understood, but it is known to involve the V-ATPase (Michaillat et al., 2012), whereas hypertonic stress is thought to involve aquaporin function and affects lateral membrane tension. In support of this model, I find that effects of mild hyperosmotic stress (e.g. 0.4 M glucose) and KOAc are additive (Figure 6e). However, excessive hypertonic stress (0.8 M glucose) masks the effect of KOAc on fission (Figure 6e). Together these results suggest that although KOAc and hypertonic stress initiate fission using different mechanisms, these two independent signaling pathways ultimately converge on one pathway that underlies the membrane fission process.

The mechanisms that drive fission are not entirely understood, but based on studies by Mayer and colleagues, a model has been proposed that involves V-ATPase function, phosphoinositide (PI) signaling and dynamin GTPase function (Zieger and Mayer, 2012; see Figure 8). Furthermore, hyperosmotic stress inactivates Ypt7, the vacuole Rab GTPase, an important contributor to membrane morphology (Brett & Merz, 2008), however it is not known if Ypt7 inactivation plays a direct role in

vacuole membrane fission. Thus, I initially tested the effect of inactivating Ypt7 on vacuole fission by treating vacuoles with Gyp1-46, a purified recombinant protein consisting of the catalytic domain of Gyp1, a GTPase-activating protein that is known to inactivate Ypt7 *in vitro* (Eitzen et al., 2000)(Albert et al., 1999). Treatment with Gyp1-46 alone (without hypertonic shock or KOAc addition) is does not induce fragmentation (Figure 7b), suggesting that Rab inactivation alone is not sufficient to drive fission. However, addition of Gyp1-46 to vacuoles treated with KOAc further enhanced fission, to the same extent as hyperosmotic shock (Figure 7b). Interestingly, Gyp1-46 did not enhance fission induced by hypertonic shock, likely because Ypt7 is already maximally inactivated under these conditions (Brett and Merz, 2008). Together, these results suggested that hypertonic shock inactivates the Rab-GTPase Ypt7, in part, to facilitate vacuole fission.

Conversion of PI(3)P to PI(3,5)P_2 by the PI5-kinase Fab1 has been shown to drive membrane fission both *in vivo* and *in vitro* (Zieger and Mayer, 2012). More specifically the ratio of PI(3)P to PI(3,5)P_2 on the vacuole membrane is thought to underlie morphology, whereby PI(3)P < PI(3,5)P_2 drives fission (Mayer et al., 2000)(Michaillat et al., 2012). Treating vacuoles with the PI3-kinase (Vps34) inhibitor wortmannin inhibits PI(3)P synthesis on membranes, causing the existing pool to be rapidly converted to PI(3,5)P_2 (Wymann et al., 1996). Thus, we next examined if addition of wortmannin could stimulate vacuole fission *in vitro*. As shown in Figure 7a, wortmannin does not affect fission induced by KOAc, suggesting that KOAc maximally stimulates PI(3,5)P_2 conversion to cause fission. Conversely, wortmannin enhanced fission induced by hyperosmotic shock (with 0.8 M glucose;

Figure 7a), suggesting that PI signaling is not targeted by hypertonic stress to induce vacuole fission.

It is worth noting that I attempted to inhibit aquaporin function with $HgCl_2$ to examine its effect on vacuole fission *in vitro*. However, Hg (II) quenches GFP fluorescence (Bozkurt and Cavas, 2009) and thus is not compatible with this assay. Furthermore, vacuoles isolated from *fps1*Δ*aqy3*Δ cells are fragmented, and thus differential centrifugation is not an effective way to quantify fission. Thus, unfortunately, I was unable to directly test whether Aqy3 plays a direct role in vacuole membrane fission using this *in vitro* assay.

Figure 6. ***Like KOAc, hypertonic shock prevents fusion and enhances fission of vacuoles in vitro***

(a) Illustration of a new quantitative, cell-free vacuole fission assay. (b) Representative fluorescence micrographs showing that our method successfully separates vacuole fragments (supernatant) from larger vacuoles (pellet) when fission is stimulated by KOAc or hypertonic shock (0.6 M sorbitol). Vacuole diameters (mean ± S.E.M.) measured by quasi-elastic light scattering are shown in the top panels. (c) Vacuole fusion (red) and fission (blue) was measured in the presence of increasing concentrations of sorbitol. (d) Progressive replacement of 125 mM KCl with KOAc enhances fission, but inhibits fusion, under isotonic condition. (e) Effect of hypertonic shock induced by sorbitol or glucose in the presence or absence of KOAc. Effect of KOAc was masked by extreme hyperosmotic shock. All data shown in c-e were normalized to isotonic conditions (***,** red); $n \geq 4$ for all experiments shown.

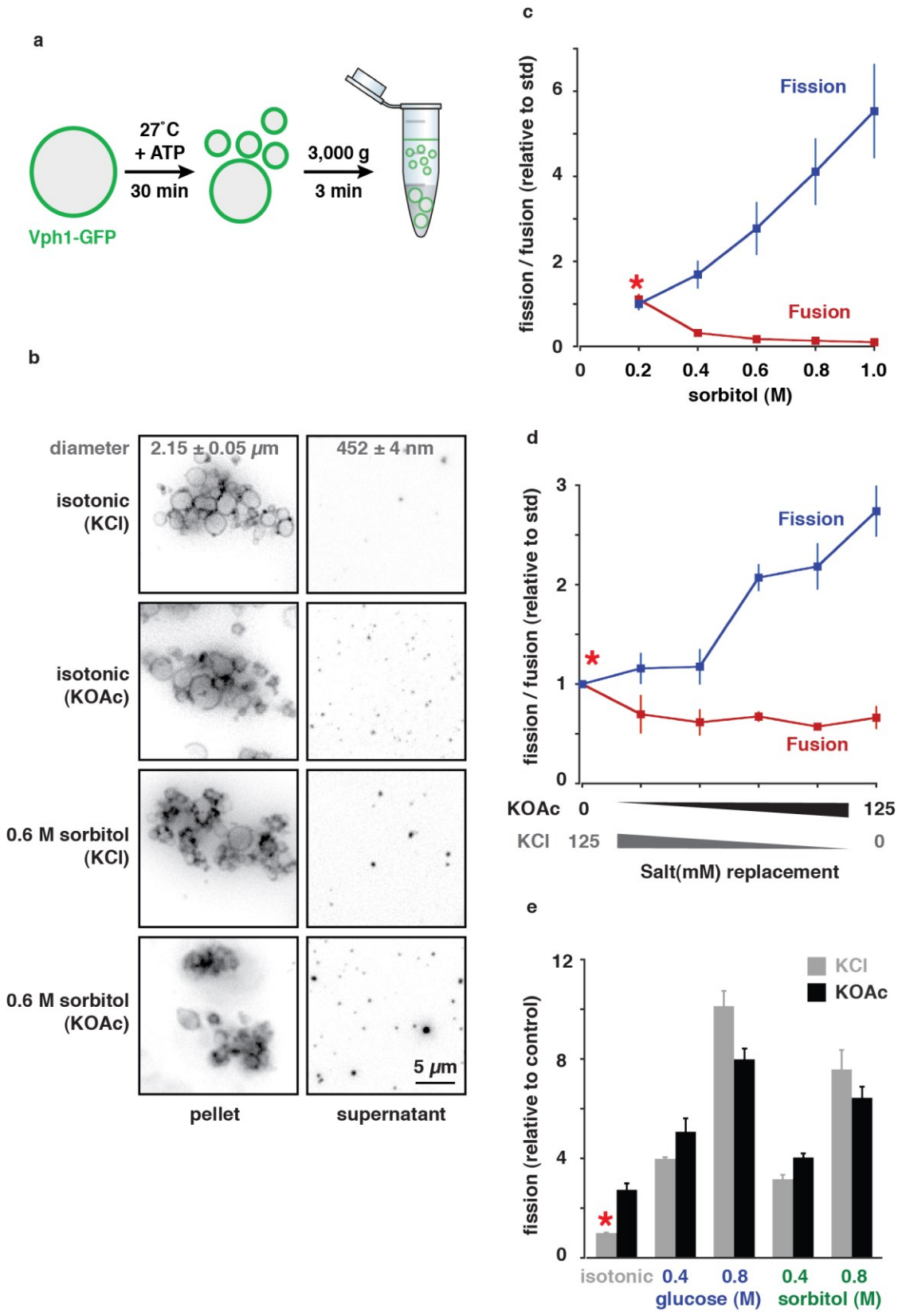
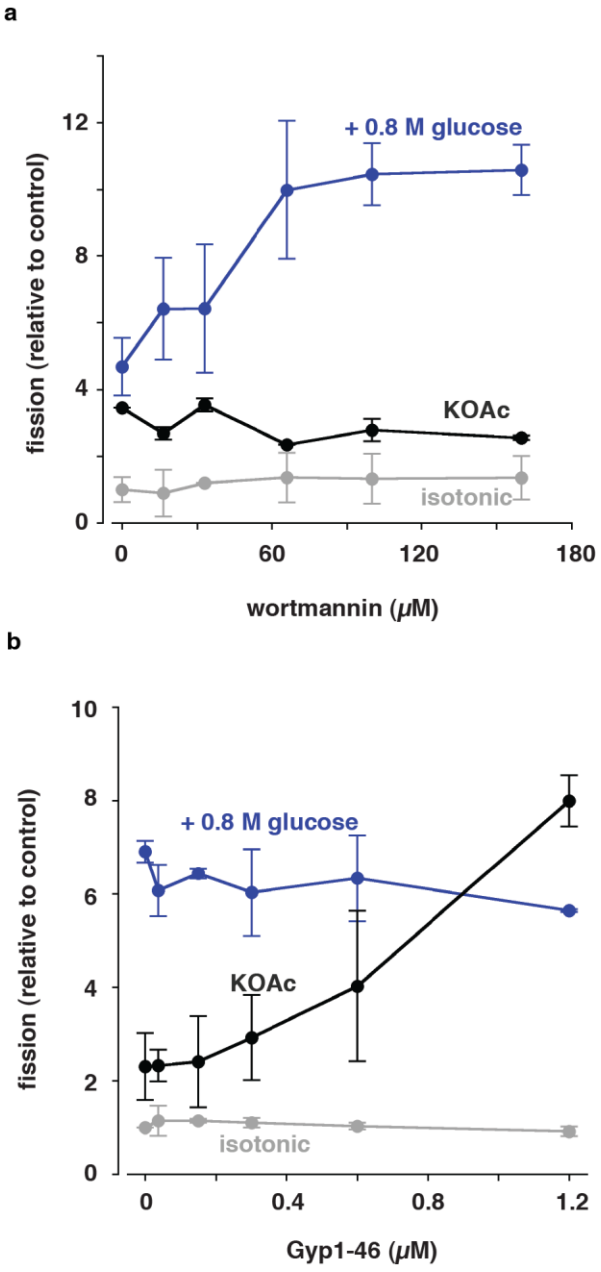

a
27˚C
+ ATP
30 min
3,000 g
3 min
Vph1-GFP
b
diameter
2.15 ± 0.05 μm
452 ± 4 nm
isotonic (KCl)
isotonic (KOAc)
0.6 M sorbitol (KCl)
0.6 M sorbitol (KOAc)
5 μm
pellet
supernatant
c
fission / fusion (relative to std)
Fission
Fusion
sorbitol (M)
d
fission / fusion (relative to std)
Fission
Fusion
KOAc 0 125
KCl 125 0
Salt(mM) replacement
e
fission (relative to control)
KCl
KOAc
isotonic
0.4
0.8
0.4
0.8
glucose (M)
sorbitol (M)

Figure 7. ***Effects of wortmannin and Gyp1-46 on vacuole fission in vitro***

Vacuole fission stimulated by KOAc or hypertonic stress (0.8 M glucose) was measured in the presence of increasing concentrations of (a) wortmannin or (b) Gyp1-46. Fission under isotonic conditions is shown as a negative control. $n \geq 4$ for all experiments shown.


a
12
8
4
0
fission (relative to control)
+ 0.8 M glucose
KOAc
isotonic
0
60
120
180
wortmannin (μM)
b
10
8
6
4
2
0
fission (relative to control)
+ 0.8 M glucose
KOAc
isotonic
0
0.4
0.8
1.2
Gyp1-46 (μM)

DISCUSSION

Under hypertonic stress, vacuoles lose water decreasing their volume to permit invagination necessary for membrane fission (see Figure 1a). Herein I show that the aquaporins Aqy3 and Fps1 mediate this observed osmosis across the vacuole membrane (Figures 2 - 4), and contribute to changes in vacuole morphology in response to osmotic stress (Figure 5). Using a new assay to measure vacuole fission in vitro, I show for the first time that hyperosmotic shock drives vacuole fission without the involvement of surface receptors and cytoplasmic signaling factors (Figure 6). Furthermore, I demonstrate that hypertonic stress inactivates Rab GTPase signaling to drive membrane fission, and this pathway is distinct from KOAc stimulated fission, but converges at later stage during the fission reaction (Figures 6 and 7), presumably where there is cross talk between Rab GTPase and PIP signaling mechanisms prior to membrane scission by Vps1 (see Figure 8). These findings reveal the mechanism(s) that control volume regulation at the vacuole, and suggest that other intracellular aquaporins may perform a similar role in regulating organelle size, shape and number in other organisms. However, one question arises from my studies: (1) How does osmosis trigger Rab inactivation required for vacuole fission? The answer to this question and a more detailed discussion of how Fps1 and Aqy3 may mediate osmosis across the vacuole membrane are given below.

How Fps1 and Aqy3 mediate osmosis across the vacuole membrane

In response to the addition of 0.9 M glucose, isolated yeast vacuoles undergo a rapid (< 0.5 second), sustained decrease in volume (Figure 2), suggesting that water quickly exits the lumen through open water channels, rather than piggyback solute transport (e.g. through the requirement of hydration required for ion translocation) by other mechanisms (Agre et al., 1993). Furthermore, of the osmolytes tested, only glucose showed a sustained decrease in volume, suggesting that the other osmolytes are transported across the vacuole membrane, depleting the osmolyte gradient required for osmosis. This was a particularly surprising result for sorbitol, as it has been shown to effectively prevent homotypic vacuole fusion and stimulate vacuole fission (Figure 7;(Brett and Merz, 2008)), but shows anemic osmosis when compared to equimolar addition of glucose (Figure 2b). However, using glucose to induce hyperosmotic shock has a greater effect on vacuole fission (Figure 7e) consistent with this observation. Furthermore, addition of glycerol does not significantly change vacuole volume (Figure 2b), consistent with previous reports that Fps1 is an aquaglyceroporin and is found on the vacuole membrane, suggesting that it may permit glycerol entry preventing rapid osmosis, and consistent with observation that glycerol is stored in the vacuole to maintain cytoplasmic osmolarity.

Also surprising was the observation that deleting only FPS1 or AQY3 nearly abolished osmosis, to the same extent as adding $HgCl_2$ (Figure 3d), i.e. deletion of either aquaporin prevent vacuole osmosis even when the other is present. One interpretation of this result is that Aqy3 and Fps1 may function as obligate hetero-

tetramers to transport water across the membrane, like many surface aquaporins studied in mammalian cells (Fetter et al., 2004). However, changes in vacuole morphology in response to osmotic stress are not impaired when FPS1 is absent, and only vacuole fission in response to hyperosmotic stress is impaired in *aqy3Δ* cells. These somewhat conflicting results, and the observation that osmosis triggered by the addition of low concentrations of sorbitol are sufficient to induce vacuole fission, have lead us to speculate that (1) only a small, rapid change in vacuole volume (observed within the first 500 ms of hypertonic shock, see Figures 2 and 3) may be sufficient to drive changes in vacuole morphology, and (2) perhaps Aqy3, and not Fps1, may directly interact the fission machinery to initiate vacuole fission.

How does osmosis trigger Rab inactivation required for vacuole fission?

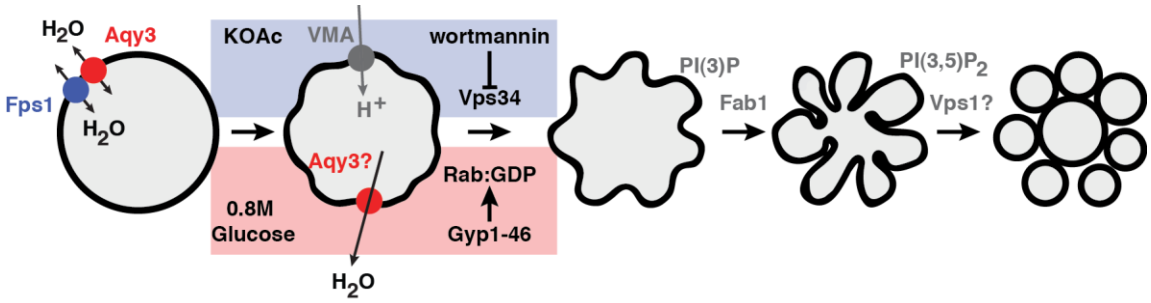
Consistent with our hypothesized roles for Aqy3 and Fps1 in osmosis, we speculate that a small rapid change in volume is sufficient to rapidly decrease lateral surface tension of the vacuole membrane. Although we have not measured this change in tension, it may be sufficient to activate the TrpY1, a mechanosensitive cation channel that opens in response to changes in membrane tension. Although it is unclear whether TrpY1 plays a direct role in the membrane fission process, it does open in response to hyperosmotic stress to mediate calcium efflux from the vacuole (Denis and Cyert, 2002). Furthermore, Ca^{2+} signaling through calmodulin and calcineurin have been shown to regulate vacuole morphology, and preliminary results from Alexey Merz's group suggest that endosomal Rab-GAP proteins may

respond to Ca^{2+} signaling (personal communication). Thus, we speculate that TrpY1 may act immediately downstream of Aqy3 in the fission pathway and that Ca^{2+} efflux by TrpY1 may trigger a Rab-GAP to inactivate Ytp7 to drive vacuole membrane fission.

To understand aquaporin function, we impose experimental conditions to drive water movement across the vacuole membrane. However, cellular osmolarity is normally tightly regulated, and vacuoles are rarely subjected to such extreme conditions in nature. Thus, to put our findings into greater biological context, we speculate that vacuole aquaporin function may be coupled to osmolyte transport in response to secondary messenger signaling that underlies changes in vacuole morphology necessary for normal yeast cell physiology. For example, yeast vacuoles must undergo fission to donate organelles to daughter cells, which is critical for organelle inheritance during cell division. As vacuoles fuse or fragment in response to changes in kinase activity, e.g. Yck3 and TorC1 (LaGrassa and Ungermann, 2005) (Michaillat et al., 2012), the possibility exists that Aqy3 (or osmolyte transporters that would stimulate osmosis through Aqy3) may respond to kinases known to orchestrate cell division, which in turn would drive water efflux required for vacuole fragmentation necessary for vacuole inheritance.

Figure 8. ***Model of mechanisms responsible for vacuole fission***

Cartoon illustrating how Aqy3 or Fps1 mediate osmosis to stimulate vacuole fragmentation upon hyperosmotic shock. Unlike, KOAc that is thought to stimulate PI-signaling through V-ATPase function, water efflux caused by hyperosmotic shock likely decreases lateral membrane tension to inactivate Ypt7 to signal downstream mechanisms responsible for membrane fission. I speculate that pathways stimulated by KOAc or hyperosmotic stress converge prior to Fab1 activity required for membrane invagination and membrane scission by the dynamin ortholog Vps1.


H2O
Aqy3
Fps1
H2O
KOAc
VMA
wortmannin
H+
Vps34
Aqy3?
Rab:GDP
0.8M
Glucose
Gyp1-46
H2O
PI(3)P
Fab1
PI(3,5)P2
Vps1?

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