Using light for energy: examining the evolution of phototrophic metabolism via synthetic construction

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Abstract

Evolutionary innovations helped transform life on Earth. The origin of phototrophy was pivotal to increasing biomass, by utilizing light-driven energy transport to drive biological processes. Retinalophototrophy constitutes one of two phototrophic pathways on Earth, consisting of a simple system of microbial rhodopsins which are exemplars of horizontal gene transfer. Here, we seek to determine if *Saccharomyces cerevisiae*, a heterotrophic fungus, can function as a facultative artificial phototroph after acquiring only one rhodopsin gene. We investigate the fitness effects of turning yeast phototrophic by using synthetic biology to insert *U. maydis* rhodopsin into the vacuole of budding yeast. We observed a selective advantage in unicellular yeast with faster growth of yeast bearing rhodopsin in the presence of green light. This simple model illustrates the capacity of horizontal gene transfer to have a large phenotypic effect, with one gene enabling the transfer of phototrophic energy production into a modern eukaryote. While subject to the constraints of any given organism, this illustrates how single horizontal gene transfer events may be used to alter ecological niches and circumvent fitness constraints.

Introduction

Major evolutionary innovations have shaped the history of life on Earth**.** Some major innovations occurred multiple times: for example, at least 50 distinct multicellular lineages have arisen from a diverse range of unicellular ancestors[1, 2]. Other transitions, such as eukaryogenesis, have evolved only once and transformed the world[3]. One of the most impactful innovations in evolutionary history is phototrophy – the ability to use light for biological energy. Originating at least 3.5 Ga ago[4], this process drives the vast majority of metabolism on Earth, directly or indirectly[5]. The ability to consume light for biological energy

has been invented twice in the history of life on Earth, with a complex history of both involving vertical as well as horizontal transfer since their inception.

The two forms of phototrophic metabolism present on Earth are chlorophototrophy and retinalophototrophy. Chlorophototrophy, observed only in a subset of bacteria including the oxygenic cyanobacteria and numerous anoxygenic phototrophs[6], uses photochemical reactions in chlorophyll and bacteriochlorophyll molecules to excite electrons to reducing potentials and either fix carbon or drive an electron transport chain to pump protons. This system is complex, with long pathways required to construct chlorophyll pigments, large membrane complexes with multiple components transducing energy, and multiple redox cofactors[5]. Retinalophototrophy utilizes a much simpler system consisting of a single microbial rhodopsin protein[7]. This small membrane protein is covalently bound to all-trans-retinal pigment and pumps a single proton per photon absorbed rather than engaging in light-driven redox reactions[8].

Microbial rhodopsins can be found in all three domains of life[9, 10], unlike chlorophototrophic reaction centers which are only found in eubacteria or eukaryotes that have taken up endosymbionts[6]. This difference is likely because of the ease of horizontal transfer of rhodopsins, which are encoded by a single gene and require only four additional genes to synthesize their appropriate pigment[8, 11-13] or even consumption of appropriate retinal pigments[14], as compared to thirty genes for a chlorophototrophic pathway[11-13]. Horizontal transfer of chlorophototrophy in the deep past has been inferred[12, 15], but is rare compared to the sheer ubiquity of horizontal transfer of rhodopsins. The simplicity and ease of transfer of microbial rhodopsins suggests that they provide an evolutionarily accessible alternative phototrophic metabolism in the face of resource-limited environments.

While horizontal transfer of this system is ubiquitous, little is known about the dynamics of horizontal transfer of this trait, and the process through which a heterotroph may be transformed into a nascent phototroph. Are organisms with no known evolutionary history phototrophy able to use this system immediately, or do they require significant adaptation before it is functional? Here, we attempt to transform *Saccharomyces cerevisiae*, a heterotrophic fungus, into a facultative artificial phototroph by introducing a fungal rhodopsin. We chose a rhodopsin localized to the vacuole, an organelle which is normally acidified via a rotary proton-pumping ATPase, thus allowing the rhodopsin's light-driven proton pump to supplement heterotrophic metabolism. By examining the physiological effects of cytoplasmic pH on starvation-triggered polymerization of metabolic proteins, we show that when supplemented with retinal pigment the cells pump sufficient protons across the vacuole membrane using light energy reduce starvation responses. By performing competition experiments, we find that rhodopsin-bearing yeast show a selective advantage over non-rhodopsin-bearing yeast in the presence of green light. Our results demonstrate that *S. cerevisiae* can be transformed into a facultative photoheterotroph without requiring evolutionary optimization or pre-adaptation.

Results

Strain construction/validation

We chose the vacuolar rhodopsin gene *UmOps2* from the fungus *Ustilago maydis*[16] to introduce into *Saccharomyces cerevisiae*. This rhodopsin localizes to the vacuole membrane, one of two membranes (the other being the inner mitochondrial membrane) in a eukaryotic cell containing a rotary ATPase theoretically capable of transducing a proton gradient into ATP. Attempts to introduce a proteorhodopsin directly from bacteria[17] resulted in localization to the

endoplasmic reticulum rather than either of these membranes. While rhodopsins have been synthetically engineered to localize to the inner mitochondrial membrane via fusions to mitochondrial inner membrane proteins[18-21], this has never been observed in nature and thus would not model a natural horizontal gene transfer event.

UmOps2 and *UmOps2-GFP* fusion genes were synthesized and codon optimized for yeast, and introduced to the yeast chromosome via a plasmid bearing homology to the silent HO locus (Figure 1A). Microscopy indicated that localization to the vacuolar compartment, previously observed in *U. maydis*, was maintained (Figure 1B). This rhodopsin is oriented to pump protons into the vacoular compartment when energized with light, a process that may either build up the vacuolar membrane potential sufficiently to run the vacuolar ATPase in reverse and generate ATP, or simply reduce ATP consumption by the vacuolar ATPase (Figure 1C), a cost which is a significant fraction of the cellular ATP budget. To absorb light rhodopsin must be covalently bound to retinal, a chromophore that converts light into metabolic energy by converting one bond from trans to cis and this shape change forcing a proton across the membrane[7, 22] (8,15). We observed that yeast bore a purple color when all-trans-retinal is added to cells containing rhodopsin, indicating that the apoprotein is able to successfully integrate this chromophore into the complex (Figure 1D).

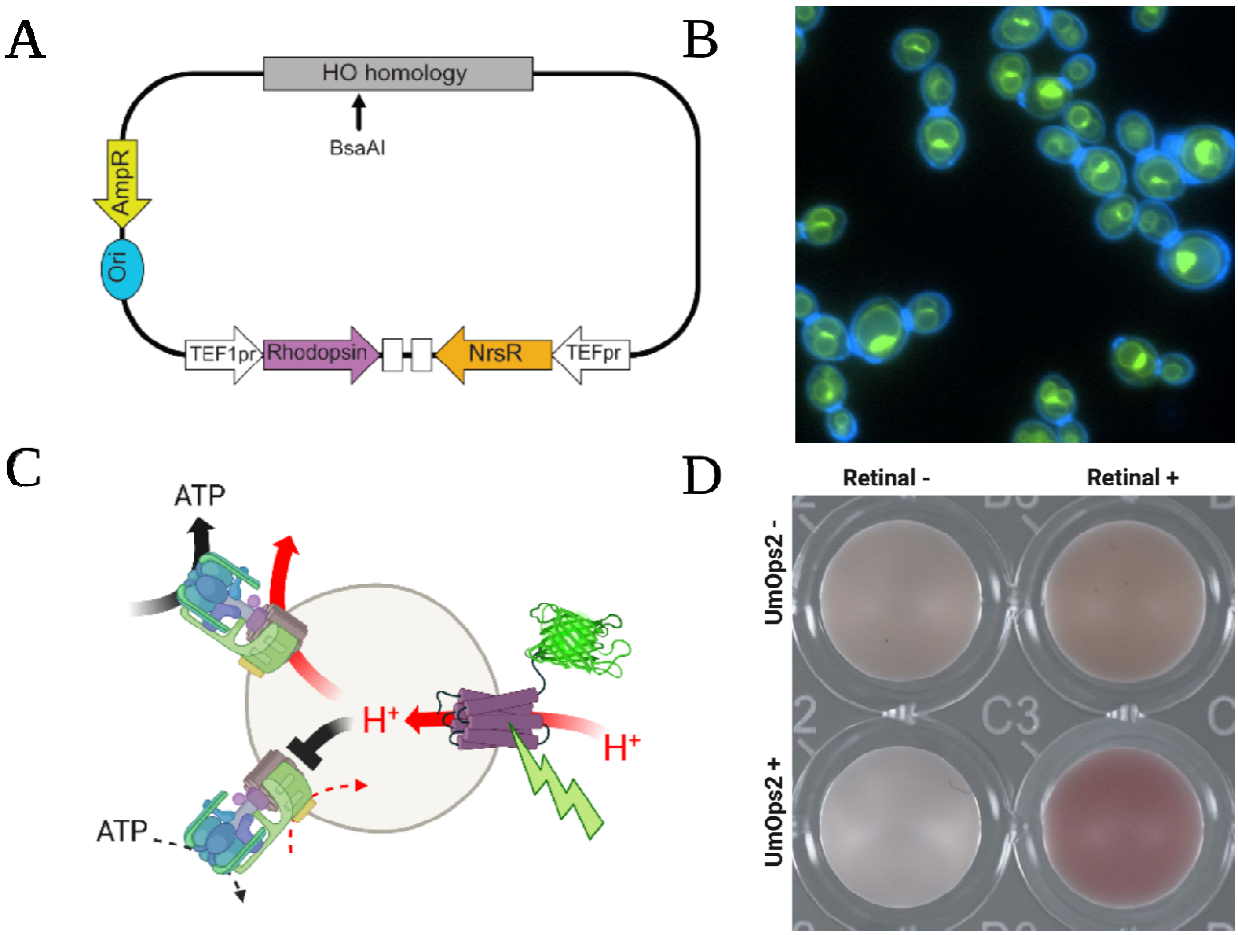

Figure 1. Construction of rhodopsin-engineered yeast. (A) *UmOps2* rhodopsin was put under the control of the *TEF1* promoter, on a plasmid with the *NatMX6* selectable marker and a segment of homology to the *HO* gene of yeast. This homology region was cut with restriction enzyme BsaAI for ends-in cloning to produce a multicopy repeat array at the HO locus. (B) UmOps2-GFP fusion protein localizes to the vacuolar membrane (cell walls stained with calcofluor in blue). (C) In the presence of green light, rhodopsin pumps protons from the cytoplasm into the vacuolar compartment. This either reduces ATP consumption by the rotary ATPase, or pushes the vacuolar membrane to a high enough voltage to allow ATP production rather than consumption. (D) Confirmation of UmOps2 protein expression and function. Yeast bearing the transgenic rhodopsin gene exhibit a purple color, but only when both the transgene is present and the yeast are supplemented with all-trans retinal.

Physiological analysis

To determine if rhodopsins are able to affect the physiology of *S. cerevisiae*, we measured the effects of green light on yeast with and without UmOps2 on the dynamics of URA7 fibers (Figure 2A). URA7 is a major CTP synthase that catalyzes ATP-dependent conversion of UTP to CTP[23] which, under conditions of starvation, polymerizes out of solution in the cytoplasm into insoluble fibers in a catalytically inactive form. This process is mediated by changes in the surface charge of the protein resulting from changes in cytoplasmic pH, and can be used as an indicator of metabolic state[24, 25]. Under ordinary circumstances as ATP production increases, the vacuolar ATPase pumps more protons out of the cytoplasm which becomes less acidic, leading to URA7 fibers dissociating into free soluble active protein (Figure 2B). This is a special case of a more broad phenomenon in which cytoplasmic pH and changes in macromolecular crowding drastically affects the physical properties of yeast cytoplasm, with starvation associated with acidic cytosol, increased protein-protein interactions, and a decrease in the function of many metabolic enzymes[26, 27].

We observed this effect of proton pumping on metabolic state via observation of GFP-tagged URA7 fibers in light and dark conditions. We grew yeast bearing *URA7-GFP* fusion genes, both with and without *UmOps2*, for 24 hours in the presence and absence of green LED illumination. Yeast bearing *UmOps2* displayed a significant decrease in visible filament area in light conditions while those without rhodopsins did not (Figure 2C; one-way ANOVA, $F_{3,16}$=9.6, p=0.0007, pairwise differences assessed with Tukey's HSD with α=0.05). The reduction in filament area demonstrates a deacidification of the cytoplasm sufficient to alter the function of metabolic enzymes in the presence of green light. While this indicates an effect on cellular physiology, and successful pumping of protons across the vacuole membrane, we do not know

whether this proton motive force is able to result in ATP synthesis. As a proton pump, rhodopsins are capable of directly deacidifying the cytoplasm even in the absence of ATP production by the vacuolar rotary ATPase. This would still likely result in reduced ATP consumption as the rotary ATPase was inhibited by high proton motive force, even if this effect does not actually result in ATP production.

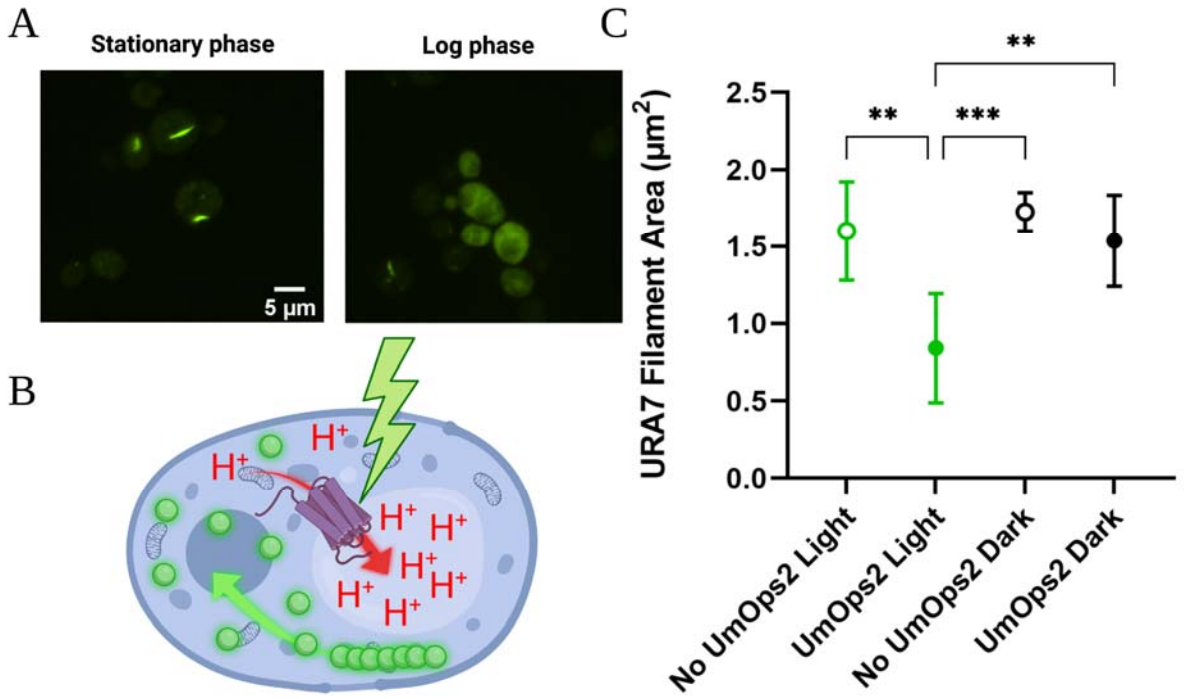

Figure 2. Examining the effect of rhodopsin function on yeast physiology. (A) Yeast bearing URA7-GFP fusion proteins exhibit a single fluorescent fiber of polymerized inactive enzyme in stationary phase, which dissociates into free active enzyme in log phase due to cytoplasmic deacidification. This can be used as a marker of physiological state, with filaments indicating starvation. (B) Model of the effect of vacuolar rhodopsin on yeast physiology. Excitation of rhodopsin with green light pumps protons from the cytoplasm to the vacuole, deacidifying the cytoplasm and dissociating URA7-GFP fibers. (C) Experimental effect of green light on URA7-GFP fiber formation in yeast with and without UmOps2 protein. Cells bearing the vacuolar rhodopsin exhibit significantly smaller URA7 fibers when grown in green light, while those without rhodopsin do not.

Darwinian fitness measurements

We measured the fitness effect of rhodopsin expression on yeast via a competition assay. Yeast were grown in Yeast Extract Peptone Glycerol media, containing a non-fermentable carbon source, supplemented with all-trans retinal. The purpose of this medium is to force respiration, and a state of low biologically available energy. When grown in YEPG, yeast cultures typically reach extremely low levels of oxygen and become oxygen-limited, respiring at a fraction of the maximum possible rate their metabolisms could theoretically support. This maximizes the potential for phototrophic energy production to supplement a low rate of respiration in these inactive cells. As rhodopsins are known to enhance survival of bacteria under conditions of starvation[28], we allowed cells to stay in stationary phase and performed transfers every 48 hours. Isolate populations were grown for 24 hours prior to the start of the competition

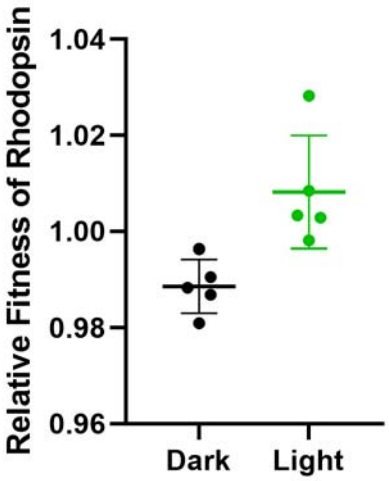

Figure 3. Selective advantage of rhodopsin-beearing yeast. Control yeast (GFP, under the same promoter as the rhodopsin strain) and yeast bearing UmOps2 were grown together in dark and light conditions for 2 passages. Rhodopsin-bearing yeast had an average 2.2% higher fitness, measured as the ratio of Malthusian growth parameters, when grown in green light compared to when they were grown in the dark.

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Control cells contained a green fluorescent protein marker to easily allow determination of genotype via flow cytometry. We measured the frequency of rhodopsin-bearing cells and calculated their selective advantage in dark and light conditions (see Methods) after 72 hours (Figure 3). The difference in fitness between light and dark conditions represents the metabolic advantage of rhodopsins, as the costs of expressing the system are controlled. We find that rhodopsins increased fitness by 2.2% in light (two-tailed *t*-test, t=2.67, n=10, p=0.017)- a modest but biologically significant advantage that would allow a rhodopsin-bearing genotype to increase from 1% to 50% frequency in just 180 generations.

Cell viability

Fundamentally, there are only two ways that a replicator can increase in frequency in a population: it can reproduce more, or die less. While rhodopsin-bearing yeast grown in light have an advantage during competition (Figure 3), it is not clear whether this benefit stems from greater reproduction or reduced mortality. To disentangle these mechanisms, we measured the viability of cells from each strain during stationary phase culture. We inoculated GFP (control) and UmOps2 bearing yeast into YEPG supplemented with retinal, and allowed them to grow for 72 hours under light and dark conditions. Every 24 hours, yeast were extracted and stained with propidium iodide to determine cell viability (Figure 4 A, B). Overall, the cell death across all populations was relatively low, ≤3%. We performed a two-way ANOVA, finding that rhodopsin or control status was significant ($F_{3,6}$=31.4, p= 0.0005), explaining 83.6% of the variation in the mortality data, as was time ($F_{2.6}$=6.18, p= 0.0349). This indicates that rhodopsin is a costly protein in our system, producing a higher rate of cell death during stationary phase. As these yeast exhibit a clear selective advantage when measured across multiple transfers (Figure 3),

however, this indicates that the selective advantage lies in enhanced growth rather than decreased cell death.

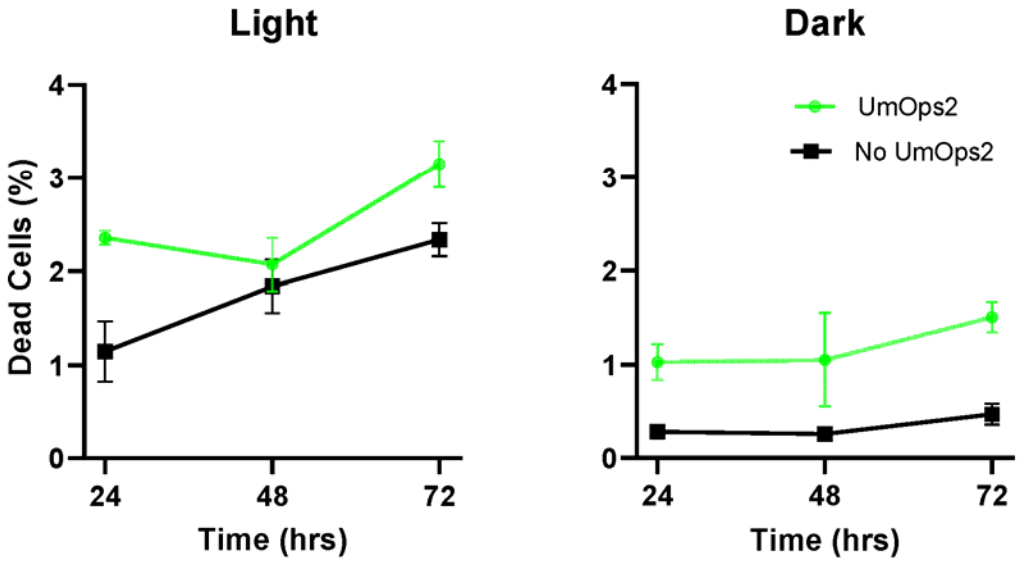

Figure 4. Effect of rhodopsin on cell viability in stationary phase. Control and UmOps2-bearing yeast were grown in YPGlycerol for 3 days in dark and light conditions, stained daily with propidium iodide to measure cell death in each population. Rhodopsin-bearing yeast in light displayed the highest cell mortality. As these cells had higher mortality while still having an overall selective advantage, their advantage in competition (Figure 3) lies in increased growth rather than reduced death.

Discussion

In this paper, we explore the ability to transform a heterotrophic eukaryote with no known history of phototrophy, *S. cerevisiae*, into a facultative phototroph. We transformed *S. cerevisiae* with a fungal rhodopsin, UmOps2, from the corn pathogen *Ustilago maydis.* UmOps2 protein localized to the yeast vacuole and provided novel function, reducing cellular starvation under

light, but had no impact on physiology in the dark. Under light, UmOps2 increased fitness by ~2.2%, which was due to increased growth rates, not reduced cellular death rates.

The success of this artificial horizontal transfer of rhodopsin from one fungal species to another underscores what we have learned from phylogenetic reconstruction: as physiologically flexible genes not dependent upon a specific organismal context to provide a selective advantage, rhodopsins are robustly capable of horizontal transfer. Previous attempts to transfer proton-pumping rhodopsins to eukaryotic species in a way that contributes to cellular bioenergentics have generally involved targeting them to the mitochondrion[18-21], the eukaryotic membrane most classically associated with ATP production, even though eukaryotic proton-pumping rhodopsins typically localize to the cell membrane, vacuole, or other endomembrane system[16, 29, 30]. These mitochondrial localizations have always involved fusion of the rhodopsin sequence to a mitochondrial inner membrane protein to successfully localize them[18-21]. This may indicate a reason that no known eukaryotic proton-pumping rhodopsins actually localize to the mitochondrion: to do so requires multiple evolutionary events to occur at once before the protein can be used usefully. A membrane protein without tuned mitochondrial localization signals will typically be integrated nonspecifically into the endomembrane system, especially the endoplasmic reticulum. It has recently been discovered that eukaryotes can obtain useful work from rhodopsins localized to the endoplasmic reticulum - diatoms bear a proton-pumping rhodopsin specifically localized to the Chloroplast Endoplasmic Reticulum Membrane (CERM) that encloses their secondary plastid, which is believed to enhance their carbon-concentration mechanism[30] in order to reduce necessary RuBisCo protein for carbon fixation. Evolution of trafficking membrane proteins to the vacuole, which involves targeting via the ALP pathway from the endomembrane system[31], would then be free to evolve gradually via mutation rather

than large-scale fortuitous gene fusions. Thus, targeting to the vacuole may be more evolutionarily accessible than targeting to the mitochondrial inner membrane, and with horizontal transfer available such faster-originating rhodopsins could come to dominate photoheterotrophic energy flux in eukaryotes via evolutionary priority effects[32-34].

Of note, while the function of UmOps2 was dependent upon supplementation with all-trans retinal, this may not represent a barrier to horizontal transfer of a complete rhodopsin system. Rhodopsins are known to occasionally transfer in multigene cassettes bearing a retinal synthesis pathway[11], and organisms with proton-pumping rhodopsins that lack retinal-production pathways are known, typically assumed to acquire it by consuming it from bacterial sources[14].

UmOps2 represents one of three described classes of fungal rhodopsins in ascomycetes - the LR-like rhodopsins which function as rapid proton-pumps as compared to CarO-like and NR-like rhodopsins which function as sensory proteins[35]. Notably, both the prototypical rhodopsin LR from the fungus *Leptosphaeria maculans* (data not shown), and the LR-type rhodopsin UmOps2 from *Ustilago maydis* are known to localize to the vacuole membrane[16]. Our results indicating that UmOps2 is capable of pumping physiologically relevant quantities of protons into the vacuole suggests that this is likely to be a general pattern, with the vacuole a eukaryotic membrane where proton pumping is most useful. Here, it can alleviate the ATP cost of acidification of the endosomal compartment or perhaps bring membrane potential high enough to run the rotary ATPase in reverse, one way or another transducing light into relevant quantities of biologically available energy. As a bioenergetically relevant membrane, it is likely that this compartment would be a common target of rhodopsins in photoheterotrophic eukaryotes.

Rhodopsin expression carries a cost in our model system. While exhibiting a selective advantage over the course of an entire competition experiment in the presence of green light, rhodopsin-bearing yeast face a selective disadvantage in the dark, and consistently exhibit a higher rate of cell death in both light and dark conditions. The reasons for this cost are unclear. It is unlikely to be a result of sheer protein biosynthesis cost, as rhodopsin and the GFP expressed in control cells are of similar mass (27 kilodaltons for GFP versus 32 kilodaltons for UmOps2), and are driven by the same promoter. However, whatever the source of this fitness cost, it is overwhelmed by the growth advantage provided by the ability to acquire energy as a photoheterotroph in the light.

Artificial photoheterotrophic metabolism may have practical applications. *S. cerevisiae* is frequently used as an industrial organism for biofuel and bioproduct production applications[36]. Photoheterotrophic metabolism stands to potentially increase their yield, as with less growth substrate needing to be fermented or burned for energy to power growth, more growth substrate can be put towards production of the desired product. This could represent an increased in yield per unit feedstock rather than necessarily an increase in the rate of growth depending on the energy flux per unit protein of rhodopsins versus glycolytic enzymes and respiratory electron transport chains[37]. This could be of interest for biofuel or bioproduct production in environments with limited material availability but high energy availability, such as the production of secondary modified metabolites or perhaps even aerospace applications.

The successful transfer of photoheterotrophic metabolism to a well-understood model organism via simple synthetic biology techniques stands to open novel research avenues. The origin of phototrophy is one of the greatest and most important transitions in the history of life on Earth, and it is likely that rhodopsin-based retinalophototrophy predates chlorophototrophy as

its earliest form[38]. Recent work has reconstructed ancient ancestors of modern rhodopsins, and probed their likely function[39, 40]. While rhodopsins have been successfully expressed in bacteria before[41, 42], a cellular environment likely more akin to the prokaryotic cells of the early Earth, many eukaryotes bear these proteins and likely have as long as they have existed[43-45]. This allows the study of the history of a fundamental biological process, phototrophy, in one of the most well-studied model organisms on Earth via the incorporation of genes from across the tree of life or even reconstructions of ancient ancestral states.

Phototrophy may also have a role to play in the evolution of multicellularity. Fossils of multicellular eukaryotic algae are observed up to 1.6 billion years ago[46], and multicellular cyanobacteria even earlier[47], long before the earliest indirect evidence of animals during the cryogenian[48]. Recent work using the multicellular “snowflake yeast” model system has shown that oxygen levels have a profound effect on the evolution of multicellular size in long-term evolution experiments, with large size evolving at low and high oxygen levels but intermediate oxygen levels[49]. These intermediate oxygen levels may correspond with the levels observed before the origin of animals but contemporaneous with the origin of multicellular algae[50]. This appears to be because at low oxygen levels, oxygen is unable to diffuse into the interior cells of multicellular clusters, giving smaller groups a fitness advantage. Could phototrophic metabolism circumvent the limiting effects of oxygen metabolism on the evolution of multicellularity by channeling another source of energy into deeper cell layers? By transferring rhodopsins to the multicellular snowflake yeast system, the role of phototrophic metabolism in the evolution of multicellularity can be probed via directed evolution in the modern day.

In summary, we have successfully transformed *Saccharomyces cerevisiae* into an artificial photoheterotroph by adding a single rhodopsin protein into the vacuole. When exposed

to green light, rhodopsin-bearing yeast had an immediate fitness advantage over controls. This required no evolutionary optimization, illustrating the ease of horizontal transfer of this phototrophic system and potentially shedding light on the evolution of rhodopsin bioenergetics and subtypes in eukaryotes. Using synthetic biology to construct photoheterotrophic organisms could have benefits for industrial purposes, as it is another method to push energy into cellular metabolism over and above chemical feedstocks. Incorporation of rhodopsins and retinalophototrophy into a well-studied model organisms opens up novel applications and research avenues, from the role phototrophy has played in the evolution of life on Earth to the early optimization of traits during the early stages of their evolution.

Methods

Engineering & Strains

The *U. maydis* rhodopsin UmOps2 reading frame was codon optimized and synthesized via ThermoFisher, as was GFP, flanked by restriction sites SpeI and AflII. Both GFP and *U. maydis* rhodopsin UmOps2 were ligated into a custom expression vector driven by a TEF1 promoter and CYC1 terminator, associated with a NAT-NT2 resistance cassette and a piece of HO gene homology bearing the loss-of-function mutations present in S288C. By cutting within this HO homology using AfeI or BsaAI, UmOps2 or GFP can be introduced as a high-copy repeat at the HO locus.

UmOps2 and GFP were introduced at high copy to the HO locus of homozygous diploid Y55 derivative Y55HD previously used by the Ratcliff laboratory[51]. These strains were sporulated, and the resultant haploid heterothallic spores mated with each other to produce

homozygous diploid control yeast homozygous for GFP at the HO locus and experimental yeast homozygous for UmOps2 at the HO locus.

GFP was fused to URA7 via PCR transformation from plasmid pYM25[52] bearing GFP and hphNT2 in the Y55HD background. Heterozygous yeast were used as a control for metabolic analysis, and after mating with yeast bearing UmOps2, double heterozygotes were used as experimental strains for metabolic analysis of the effect of rhodopsin on URA7 fiber morphology.

Cell Culture and competition.

All yeast were grown in liquid Yeast Extract Peptone Glycerol (YEPG) media supplemented with 10 microliters of 10 mM all-trans retinal in 100% ethanol, for a final concentration of 10 µM of all-trans retinal. This supplemented medium was used for all steps.

For competition experiments, control and rhodopsin bearing yeast were grown separately to saturation shaking at 250 RPM at 30 °C for 24 hours. On Day 0, 500 µL of both GFP-bering controls and rhodopsin-bearing cells were combined and mixed. This mixture was measured via flow cytometry to determine the baseline ratio of control and rhodopsin-bearing yeast. 50 µL of mixed stationary-phase culture was transferred two new tubes of retinal-supplemented YPG. One was placed in an ordinary shaking incubator at 250 RPM and 30 °C, while another was placed in another shaking incubator with the same settings supplemented with green LEDs and a reflective foil wrapping to provide bright green illumination, measured at an average intensity of 1996 lux. These cells were allowed to grow for 48 hours, upon which 50 µL of stationary phase culture was transferred to new tubes and placed into the same incubator. At day 1 and day 3, samples were taken for flow cytometry to count GFP and rhodopsin bearing yeast.

Fitness quantification

Frequency of different cell types were determined either via flow cytometry or microscopy, as described above. Relative fitness can be determined from the change in the frequency genotypes over time. The relative fitness W of a rhodopsin-bearing yeast can be defined as the ratio of the number of generations of rhodopsin-bearing yeast (G_R) divided by the number of generations of a control yeast (G_C) over the same time frame - see Equation 1.

$$\text{Equation 1: } W = \frac{G_R}{G_C}$$

The number of generations experienced by a genotype in a population (G) after a number of transfers n during which the population reaches saturation can be calculated from the fraction of the population at passage (F_n), the fraction of the population at passage 0 (F_0), the dilution factor (d), and the number of transfers (n) using Equation 2:

$$\text{Equation 2: } \mathrm{G} = log_2 \left(\frac{F_n}{F_0} d^n\right)$$

By combining Equation 1 and Equation 2, we determine the equation for relative fitness W in terms of number of passages (n), fraction of the population bearing rhodopsin after passage n (Fr_n) and at passage 0 (Fr_0), fraction of the population that are controls after passage n (Fc_n) and at passage 0 (Fc_0), the dilution factor (d), in the form of Equation 3:

$$\text{Equation 3: } W = \frac{log_2 \left(\frac{fr_n}{fr_0} d^n\right)}{log_2 \left(\frac{fc_n}{fc_0} d^n\right)}$$

Equation 3 was used to calculate the relative fitness of rhodopsin-bearing yeast in dark and light incubation for all 5 replicate populations each at day 3 of cultivation, such that dilution factor d equals 200 and number of passages n equals 2.

Cell Viability

In order to quantify the viability of cells, a propidium iodide stain was used to measure the proportion of dead cells as a rhodopsin-bearing population and control population progressed through stationary phase**.** 5 replicate populations were inoculated and grown in YP Glycerol supplemented with all-trans retinal for 72 hours. After 24, 48, and 72h, 500 uL of each population was spun down and double washed, resuspending in 1 mL of water for imaging. 1 µL of propidium iodide stock solution (1 mg/mL) was added to the pellet and was imaged on a TI Nikon Eclipse Ti-Motorized Inverted Microscope. Four fields of view were imaged at 20x objective magnification, capturing a bright-field image, and an image with TRITC filter and 1.3 ms exposure to capture propidium iodide stained dead cells. Images were thresholded using MaxEntropy auto thresholding in Fiji Image Analysis to identify and count dead cells, and the bright-field channel segmented via Cellpose[53] to count total cells.

URA7 Filament Metabolic Analysis

In order to determine whether rhodopsin provides an energy benefit, metabolic state was assessed via polymerization of GFP-tagged URA7p. 50 µL control yeast bearing GFP-tagged URA7p, and experimental yeast bearing this fusion and the *UmOps2* gene, were inoculated and grown for 24 hours in 10 mL Synthetic Complete media supplemented with all-trans retinal. Five replicates each were grown in a dark incubator at 250 RPM and 30 °C, and a green illuminated incubator at 30 °C with light approximately 1996 lux. At 24 hours growth, stationary phase cells were removed and quickly imaged on a TI Nikon Eclipse Ti-Motorized Inverted Microscope. Fibers were captured in the FITC channel with a constant exposure of 1s and gain of 64.0x . The

average filament area was quantified using Fiji Image Analysis via automatic image segmentation using MaxEntropy auto thresholding of particles ≥ 10 pixels in size.

Strains used:

yAB406: Y55HD background, *ho:UmOps2:NatMX6/ho:UmOps2:NatMX6*

yAB414: Y55HD background, *ho:UmOps2:NatMX6/ho:UmOps2:NatMX6*

yAB452: Y55HD background, *URA7/URA7-GFP:HphNT2 HO/ho:GFP:NatMX*6

yAB703: Y55HD background, *ho:GFP:NatMX6/ho:GFP:NatMX6*

Plasmids used:

pYM25: GFP for protein fusion and a hphNT2 resistance cassette[52]

pWR112: UmOps2 under a TEF1 promoter, NATMX6 resistance, HO homology for chromosomal insertion

pWR113: Expression of UmOps2-GFP under a TEF1 promoter, NATMX6 resistance, HO homology for chromosomal insertion

pWR162: Expression of GFP under a TEF1 promoter, NATMX6 resistance, HO homology for chomosmal insertion

Oligonucleotides used for URA7-GFP fusion:

Ura7_S2:

TAGAAATTTATCTTTGTTAATGCAGTAGACTTTTAATTCTAAAATTTTGATTTAATCG ATGAATTCGAGCTCG

Ura7_S3:

GTCATTGAAGGTAAGTACGATCTTGAGGCCGGCGAAAACAAATTCAACTTTCGTACG

CTGCAGGTCGAC

Author Contributions

AB conceived of the research. AP, AB and WCR planned the experiments. AP and AB performed the experiments and analyzed the data. All authors contributed to writing. This work was supported by a NSF Graduate Research Fellowship to AP, and NSF grant DEB-1845363, Human Frontiers Grant RGY0080/2020 and a Packard Foundation Fellowship for Science and Engineering to WCR.

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