FEBS Letters 554 (2003) 194–200 FEBS 27788

Photoconversion of matrix targeted GFP enables analysis of continuity and intermixing of the mitochondrial lumen[1]

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Received 4 August 2003; accepted 6 October 2003

First published online 16 October 2003

Edited by Vladimir Skulachev

Abstract **We establish photoconversion of green fluorescent protein (GFP) as an optical 'highlighter' to investigate the continuity of the mitochondrial matrix in living budding yeast (*Saccharomyces cerevisiae*). Photoconversion of GFP resulting in a marked shift of the absorption and emission spectra to longer wavelengths is elicited, under low oxygen conditions, by irradiation with blue light. Photoconversion induced a several 100-fold increase in red fluorescence of matrix targeted GFP without affecting cell viability. The color changing facilitates simple and effective regional optical marking in a conventional fluorescence microscope. We found the mitochondrial compartment of *S. cerevisiae* to generally consist of one luminally continuous large part and occasionally some additional smaller fragments. Separated fragments fuse within a few minutes to the large part, resulting in a rapid intermixing of the entire mitochondrial matrix compartment. In Δ*fis1* and Δ*dnm1* mutants restricted in outer membrane fission, the mitochondria are still luminally continuous, suggesting a tight coupling of inner and outer membrane fissions. Matrix constrictions frequently occurring in wild type cells as well as in Δ*fis1* and Δ*dnm1* mutants do not interfere with luminal continuity.**

Key words: Drp1; Membrane fission; Mitochondrial dynamics; Photoactivation; Photoinduction; *Saccharomyces cerevisiae*

1. Introduction

Mitochondria are highly dynamic double membrane bounded organelles that constantly change their shape by tubule fissions and fusions [1–4]. Recently it has been demonstrated for several mammalian cell lines that their mitochondria are luminally discontinuous and functionally heterogeneous [5]. Although electron and fluorescence microscopy suggested a single ramified large mitochondrion in logarithmically growing budding yeast cells [6–8], no direct evidence for a continuous mitochondrial network in yeast at a given point in time has been provided.

The level of co-regulation of inner and outer membrane fissions is anticipated to have strong influence on the functional homogeneity of the mitochondrial compartment in a single cell. Inner membrane fissions without concomitant outer membrane fissions would result in isolated matrix fragments enveloped by a common outer mitochondrial membrane. In this case solute diffusion within the mitochondrial network would be largely inhibited. Such a septated mitochondrion would be contradictory to the idea of mitochondrial structures being an electrical continuum [9].

The dynamin related GTPase Dnm1p and Fis1p are crucial components of the mitochondrial fission machinery in yeast [1,2,4,10–12]. As outer membrane proteins, they are likely involved in outer membrane fission. The components of the inner membrane fission apparatus are less well described [13]. Moreover, it is unclear how and to what extent the machineries for inner and outer membrane fissions interact.

To investigate the luminal continuity of the mitochondrial compartment in wild type cells and in mutants lacking Dnm1p and Fis1p we decided to employ green fluorescent protein (GFP) as an optical 'highlighter'. Several approaches have been described to photochemically modulate fluorescent proteins. Such optical 'highlighter' can be marked by brief, localized, intense illumination and then tracked in space and time (for review see [14,15] and references therein). Photoconversion (also termed photoinduction) of GFP resulting in a shift of the absorption and emission spectra to longer wavelengths upon strong illumination with blue light has been one of the first examples of such a 'highlighter' [16,17]. With the exception of one study analyzing the mobility of photoconverted GFP in bacteria, the potential of photoconversion to study protein dynamics in living cells has not been exploited [18]. Likely three reasons can be cited for this: First, GFP photoconversion requires low oxygen conditions. Second, there have been conflicting reports on the nature of the photoconverted fluorophore [19]. And third, photoconverted GFP is allegedly very dim [20].

In this study it is demonstrated that the required low oxygen conditions, that can be regarded as physiological for the facultative anaerobe *Saccharomyces cerevisiae*, are no obstacle for imaging living budding yeast. We provide evidence that the red fluorophore generated by photoconversion is a photomodulated GFP, and demonstrate that it is very bright under the experimental conditions employed. Photoconversion of GFP is used to investigate the continuity and the intermixing of the mitochondrial matrix in *S. cerevisiae*. The findings suggest a tight co-regulation of the fission machineries of inner

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[1] Supplementary data associated with this article can be found at doi:10.1016/S0014-5793(03)01170-0.

Abbreviations: GFP, green fluorescent protein

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01170-0

and outer membranes, which appear to be independent of the apparatus performing matrix constrictions.

2. Materials and methods

2.1. Yeast strains and growth conditions

Growth and manipulation of yeast was carried out according to standard procedures [21]. Cells were routinely grown in yeast extract–peptone–glucose medium (YPD) (1% yeast extract, 2% peptone, 2% glucose), in peptone–minimal glucose medium (PMGlu) (1.7 g/l yeast nitrogen base (Difco, USA), 20 mg/l adenine sulfate, 5 g/l ammonium sulfate, 5 g/l peptone 140 (Gibco BRL, UK), 2% glucose) or in peptone–minimal galactose medium (PMGal) (as PMGlu but 2% galactose) at 30°C. All strains used in this study were isogenic to BY4741 or BY4742. Deletion strains were obtained from EUROSCARF (Frankfurt, Germany). Disruptions were confirmed by polymerase chain reaction.

To label the mitochondrial matrix with GFP from *Aequorea victoria* containing the Ser65Thr mutation [22] cells were transformed with pVT100U-mtGFP. The plasmid contains a DNA fragment encoding GFP fused to subunit 9 (amino acids 1–69) of the F_0-ATPase of *Neurospora crassa* under control of the constitutive alcohol dehydrogenase promoter [23].

2.2. Photoconversion and imaging

For photoconversion cells were placed under a coverslip and embedded in 1% low melting agarose in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). The oxygen concentration was reduced either by mitochondrial respiration of the cells themselves, or by adding a coupled glucose oxidase–catalase system (0.25 mg/ml glucose oxidase (Sigma), 0.06 mg/ml catalase (Sigma), 50 mM glucose) to remove oxygen from the medium enzymatically.

Photoconversion of GFP in entire cells was induced by illuminating the GFP expressing cells for 1–20 s with blue light in an epifluorescence microscope (Leica DMIRE2) via the fluorescein filter set. The microscope was equipped with a HBO 50 W mercury lamp. The light intensity in the sample was $\sim$7 W cm^{-2}.

Spot photoconversion was performed with a beam scanning confocal microscope (Leica TCS SP2, Leica Lasertechnik, Heidelberg, Germany). For spot photoconversion GFP labeled mitochondria were first visualized using very low light intensities for GFP excitation (70 W cm^{-2} in the focus, scanned rapidly over the sample). Low light imaging did not induce observable photoconversion. Subsequently a spatially confined area was illuminated with focused laser light (488 nm, $\sim$30 kW cm^{-2} focused to a diffraction limited spot). Spot photoconversion was accomplished by scanning $\sim$20 times with 400 Hz over the same area. The photoconverted GFP was excited at $\lambda = 543$ nm and detected at 570–630 nm.

For all image acquisition a beam scanning microscope (Leica TCS SP2, Leica Lasertechnik, Heidelberg, Germany) equipped with a 1.2 numerical aperture water immersion lens (Leica 63×, Planapo) or a 1.4 numerical aperture oil immersion lens (Leica 100×, Planapo, Wetzlar, Germany) was employed. For fluorescence detection photo-

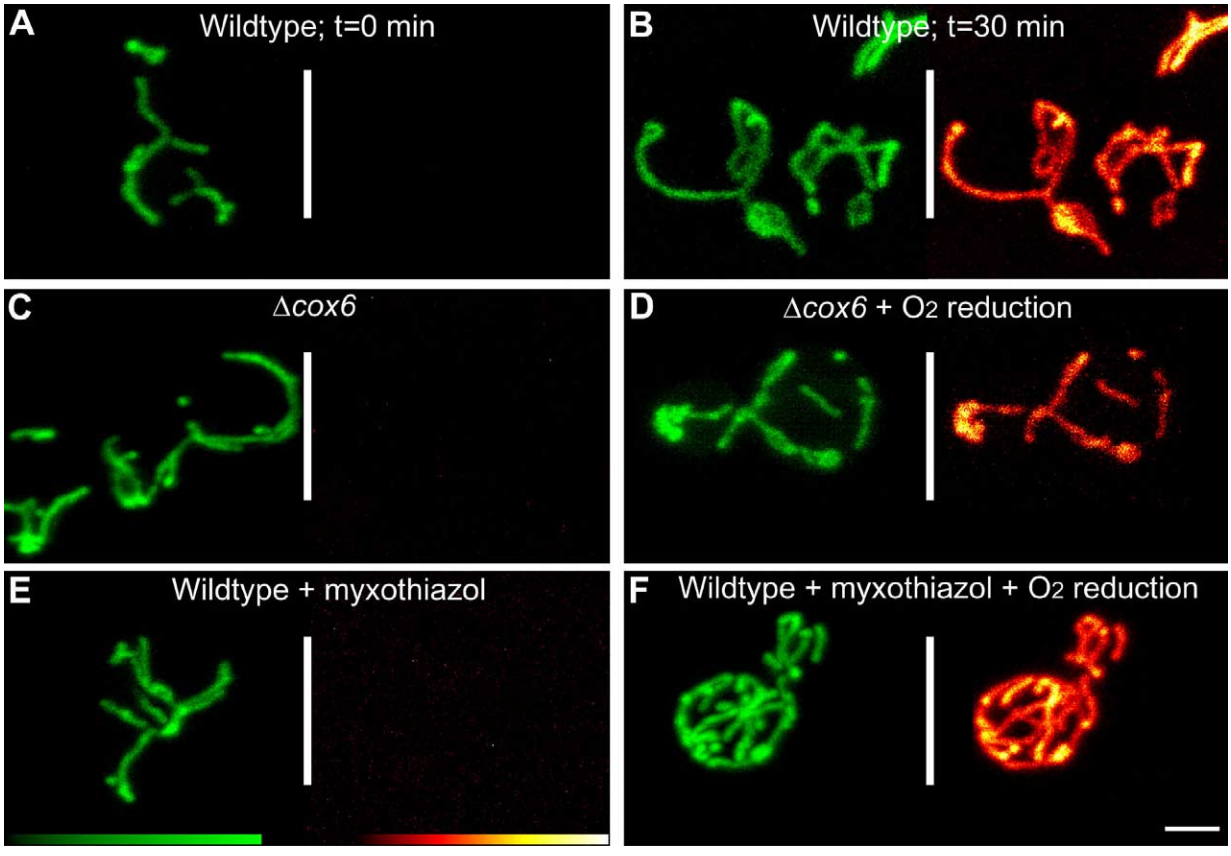

Fig. 1. Low oxygen concentrations are a prerequisite for photoconversion of GFP. Budding yeast cells expressing matrix targeted GFP were grown to logarithmic growth phase and embedded in 1% low melting agarose. In each panel on the left (green look up table): Green fluorescence of GFP ($\lambda_{exc} = 488$ nm; $\lambda_{detection} = 500$–530 nm). On the right (fire look up table): Red fluorescence of photoconverted GFP ($\lambda_{exc} = 543$ nm; $\lambda_{detection} = 570$–630 nm). A: Wild type cells imaged directly after embedding display green fluorescence, but GFP is not photoconvertable by illumination with blue light (10 s, fluorescein filter set, 7 W cm^{-2} in the sample). B: Mitochondrial targeted GFP in wild type cells after 30 min under the coverslip can be photoconverted. C: No GFP photoconversion in Δ*cox6* mutants deficient in mitochondrial respiration is observed after 30 min. D: GFP photoconversion in Δ*cox6* mutants in the presence of an oxygen scavenging system. E: No GFP photoconversion in wild type cells inhibited in mitochondrial respiration by 10 μM myxothiazol after 30 min. F: GFP photoconversion in wild type cells in the presence of 10 μM myxothiazol and an oxygen scavenging system. Displayed are maximum intensity projections of optical planes imaged with a laser scanning confocal microscope. Bar: 2 μm.

multiplier tubes or avalanche photodiodes were used. All imaging was performed at ambient temperature (~22°C).

2.3. Fluorescence lifetime determination

The lifetime of the photoconverted GFP was determined by time correlated single photon counting employing a confocal fluorescence microscope as described previously [24]. In brief, after photoconversion of matrix targeted GFP in living budding yeast cells the fluorophore was excited with $\lambda = 555$ nm pulses of ~300 fs duration from an optical parametric oscillator (APE GmbH, Berlin, Germany). Fluorescence was detected with a photon counting detector (SPCM-AQR-13, Perkin-Elmer, Fremont, CA, USA) and a TCSP card (SPC 535, Becker and Hickl GmbH, Berlin, Germany).

2.4. Protein purification

The coding sequence of GFP was excised with *Bam*HI and *Eco*RI from pYES-mtGFP [23] and subcloned into the pRSETA vector (Stratagene) that was predigested with *Bam*HI and *Eco*RI to create pRSETA-GFP. This plasmid was transformed into *Escherichia coli* BL21CodonPlus (Stratagene). For protein expression, cells carrying the recombinant plasmid were grown to an OD_{600} of 0.6 in Luria–Bertani medium (LB) containing 100 μg/ml ampicillin and were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Following 6 h incubation at 25°C the resultant biomass was pelleted by centrifugation, resuspended in PBS with protease inhibitors and sonicated. The bacterial lysate was cleared by centrifugation, and GFP was affinity purified from the supernatant on a Ni-nitrilotriacetic acid (NTA)-agarose column (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The affinity purified GFP was further purified using size exclusion chromatography on a Superdex 200 pg column (Amersham Pharmacia). The purity of the obtained protein fractions was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent silver staining.

3. Results and discussion

3.1. Low oxygen conditions are required for photoconversion of GFP

To investigate the requirements of photoconversion in *S. cerevisiae* wild type yeast cells expressing mitochondrial targeted GFP were embedded in low melting agarose under a coverslip. Under these conditions the cells displayed vivid green fluorescence (Fig. 1A). Illuminating the cells for 10 s with blue light (fluorescein filter set; 450–490 nm excitation;

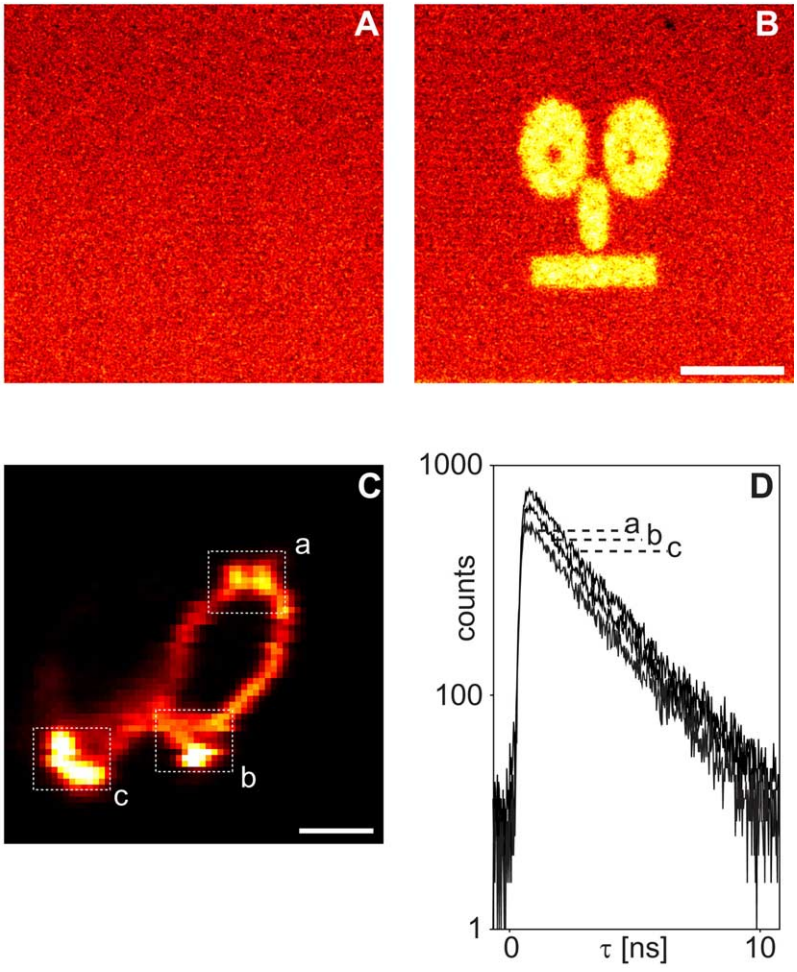

Fig. 2. The red fluorophore is photoconverted GFP. A, B: GFP was expressed in *E. coli* and purified to homogeneity. The proteins were spread on a coverslip and overlaid with PBS and an oxygen scavenging system. A: Red fluorescence of GFP before photoconversion ($\lambda_{exc} = 543$ nm; $\lambda_{detection} = 570$–$630$ nm). B: Red fluorescence after photoconversion. Imaging conditions were identical to A. Photoconversion was accomplished by scanning with a focused intense 488 nm laser beam over a spatially confined area. Bar: 5 μm. C, D: Fluorescence lifetime microscopy of photoconverted GFP. Living budding yeast cells expressing matrix targeted GFP were embedded in PBS with 1% agarose for 30 min. Following photoconversion with blue light the lifetime of the photoconverted GFP was determined by time correlated single photon counting. C: Fluorescence intensity image. Displayed is a single optical section. D: Corresponding fluorescence curves integrated over the designated areas. A lifetime of 2.11 ± 0.05 ns for the photoconverted GFP was obtained by measuring it at 25 locations in 10 cells. Bar: 2 μm.

7 W cm^{-2} in the sample) directly after embedding in agarose did not induce observable red fluorescence excitable at 543 nm (Fig. 1A). Respiration competent yeast cells consume molecular oxygen from the medium below the oxygen concentration threshold required for photoconversion within less than 30 min. Following photoconversion the mitochondrial network displayed a bright red fluorescence (emission maximum ~600 nm), which co-localized with the green fluorescence (Fig. 1B). To prove that low oxygen levels are necessary for photoconversion we utilized yeast cells with diminished mitochondrial respiration. Mutant cells lacking *COX6*, coding for a component of the respiratory chain complex IV, have an impaired mitochondrial respiration. In addition, the respiration chain of wild type cells was pharmacologically inhibited with 10 μM myxothiazol (inhibitor of complex III) or with 2 mM potassium cyanide (inhibitor of complex IV; data not shown). Mitochondrial targeted GFP of these cells was not photoconvertable, if cells were placed for 30 min under a coverslip (Fig. 1C and E). Upon enzymatic removal of oxygen from the mounting medium with a coupled glucose oxidase–catalase system GFP was readily photoconvertable (Fig. 1D and F). Although additional factors cannot be excluded these findings prove that low oxygen concentrations are important for photoconversion.

3.2. Red fluorophore is photoconverted GFP

Previously the red fluorophore observed after photoconversion has been reported to be a porphyrin derivative [19]. However, purified GFP spread on a coverslip and incubated with an oxygen removing system could readily be photoconverted in vitro using laser light of $\lambda = 488$ nm in a laser scanning microscope (Fig. 2A and B; and see [16]). Our purification protocol, involving affinity chromatography and size exclusion chromatography should have effectively removed porphyrins from the sample, making the involvement of porphyrins in case of the in vitro photoconverted GFP unlikely. Furthermore, we measured the lifetime of the photoconverted GFP in mitochondria of living cells as 2.11 ± 0.05 ns with time correlated single photon counting (Fig. 2C and D). This is close to the ~2.4 ns lifetime of the green fluorescence of GFP [24]. The lifetime of the red fluorophore is distinct from the previously published lifetime of 15.5 ± 0.1 ns, which is in the range of porphyrins [19]. This excludes porphyrins as the red fluorophore in the photoconverted mitochondria. We conclude that a photochemically converted GFP is responsible for the observed red fluorescence.

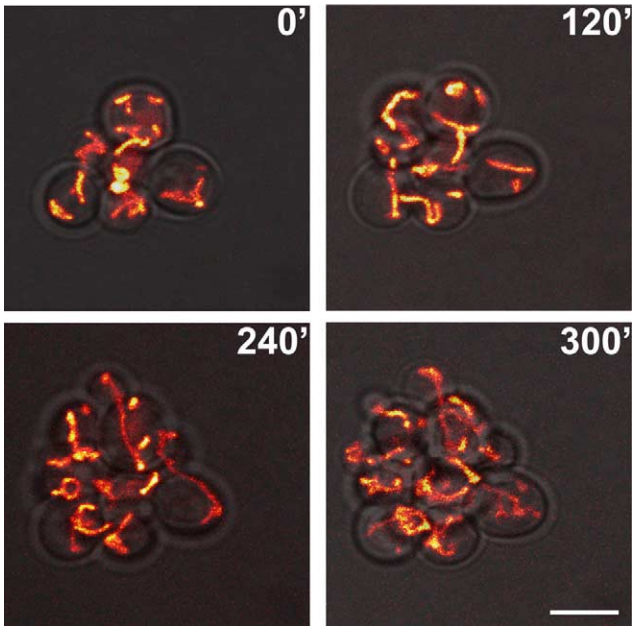

Fig. 3. Photoconversion of matrix targeted GFP does not interfere with cell viability. Wild type cells expressing matrix targeted GFP were grown until logarithmic growth phase and placed on a thin layer of YPD agar. Cells were covered with a coverslip. After 2 h GFP was photoconverted by illumination with blue light for 10 s (fluorescein filter set, 50 W Hg lamp). The red fluorescence of the photoconverted GFP was imaged with a laser scanning confocal microscope at the indicated time points (min). Displayed are maximum intensity projections overlaid with a bright field image. This time-lapse sequence is also displayed in Movie 1. Bar: 5 μm.

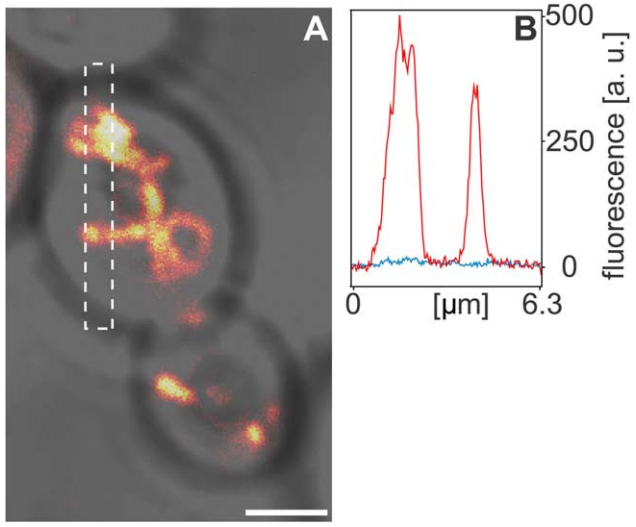

Fig. 4. Increase of red fluorescence upon photoconversion. Wild type cells expressing matrix targeted GFP were embedded in 1% agarose for 30 min. Red fluorescence ($\lambda_{exc} = 543$ nm; $\lambda_{detection} = 570$–630 nm) was recorded before and after photoconverting GFP by illuminating the cells for 10 s with blue light (fluorescein filter set, 50 W Hg lamp, 7 W cm^{-2} in the sample). A: Overlay of the photoconverted GFP fluorescence with a bright field image. B: Pixel intensities of the fluorescence of the photoconverted GFP added along the *x*-axis within the box indicated in A. Blue graph: Red fluorescence before photoconversion. Red graph: Red fluorescence after photoconversion. Bar: 2 μm.

3.3. Photoconverted GFP is stable and does not interfere with cell viability

Sufficient nutrients provided facultative anaerobic budding yeast cells grow and divide when sealed under a coverslip. Under these conditions they lower, through mitochondrial respiration, the oxygen concentration below the threshold necessary for photoconversion. By illuminating cells expressing matrix targeted GFP with a short (~10 s) pulse of blue light (fluorescein filter set) GFP was partially photoconverted. This vivid color change persisted in living cells for at least 5 h (Fig. 3 and Movie 1). Following photoconversion the cells underwent several cell divisions and the photoconverted GFP was transferred from the mother cell to the bud. Hence the low oxygen conditions are physiological for these cells. No signs of reduced cell viability were observed following photoconversion.

3.4. Photoconversion induces a several 100-fold increase in red fluorescence

In living cells a several 100-fold increase in red fluorescence was induced by a 10 s blue light illumination pulse (50 W Hg lamp, fluorescein filter set, 7 W cm^{-2} in the sample) (Fig. 4). The estimation of a several 100-fold increase is based on the increase of the mean pixel intensities of the red fluorescence of mitochondrial targeted GFP upon photoconversion. This estimation represents a lower limit of the effective increase of red fluorescence after photoconversion, because its determination was ultimately limited by (the low) background noise and possibly by cellular autofluorescence.

Since the red fluorescence of photoconverted GFP can be completely separated from the green fluorescence of non-photoconverted GFP, photoconversion results in a marked optical contrast under physiological conditions. In contrast to approaches using fluorescence recovery after photobleaching (FRAP), which require almost complete bleaching of the fluorophore, partial photoconversion is sufficient to follow the dynamics of the converted fluorophore. We employed photoconversion to explore the continuity of the mitochondrial matrix.

3.5. Mitochondrial matrix of wild type cells is continuous

For GFP targeted to the mitochondrial matrix of mammalian cells a diffusion coefficient of $2–3\times10^{-7}$ cm^2 s^{-1} has been measured [25]. Because GFP and its photoconverted red emitting fluorescent state have comparable diffusion coefficients in bacteria [16,18] it is reasonable to assume a comparable diffusion coefficient for the photoconverted GFP in budding yeast mitochondria. In case of a luminally continuous mitochondrial network, photoconversion of a small area of a GFP labeled mitochondrion would result in a homogeneous distribution of the photoconverted GFP within the experimental timeframe of a few seconds.

We photoconverted matrix targeted GFP in a small fraction of the network (see blue circle in Fig. 5A). About 5 s later (the minimal time required to switch the filters in the microscope) the distribution of the photoconverted fluorophore was imaged (Fig. 5B). Subsequently the whole network was visual-

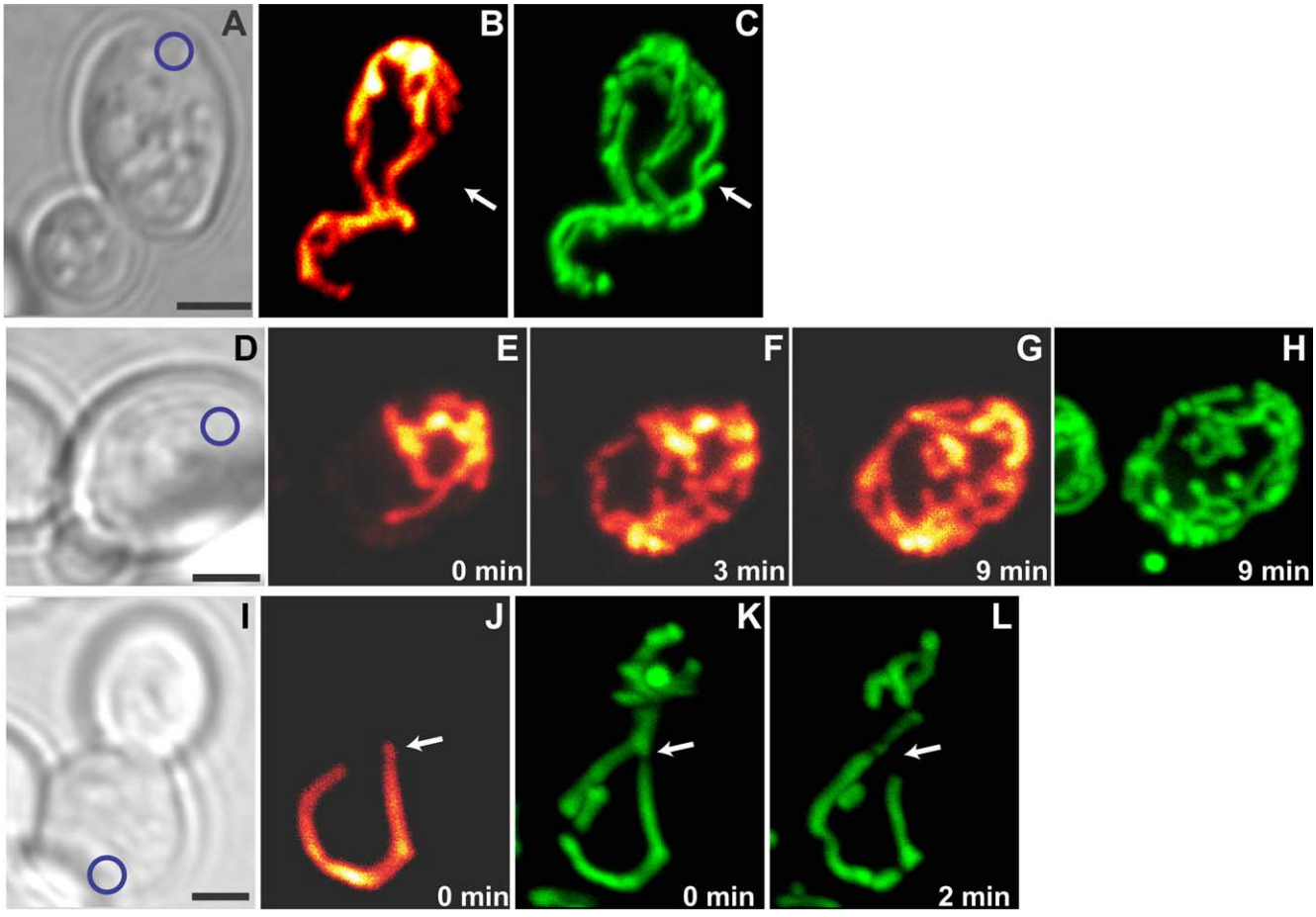

Fig. 5. Spot photoconversion of matrix targeted GFP expressed in wild type cells. A–C: Spot photoconversion on wild type cells expressing matrix targeted GFP after a 30 min incubation period under the coverslip to lower the oxygen concentration. A: Bright field image. The blue circle indicates the area of photoconversion. B: Photoconverted GFP. C: Green GFP fluorescence. The arrows indicate a tubule that is luminally discontinuous with the main mitochondrial network. D–H: Intermixing of the mitochondrial matrix in wild type cells. D: Bright field image. E–G: Distribution of the photoconverted GFP imaged at the indicated time points. H: Green fluorescence of GFP recorded after 9 min. Note that the mitochondrial matrix of the cell on the left is discontinuous to the matrix of the right cell. I–L: Time-lapse imaging of a seemingly continuous but luminally discontinuous mitochondrial network of a wild type cell. I: Bright field image. J: Photoconverted GFP. K: Green GFP fluorescence imaged immediately after recording of J. L: Green GFP fluorescence imaged 2 min later. The arrows indicate a site that was initially seemingly continuous but luminally discontinuous. Displayed are maximum intensity projections. The intermixing of the matrix (D–H) is also displayed in Movie 2. Bars: 2 μm.

ized by imaging the distribution of the remaining unconverted GFP (Fig. 5C). We found that around 50% of the logarithmically growing cells contained a luminally continuous mitochondrial network ($n > 25$). In the remaining cells the point photoconverted GFP diffused into 30–95% of the mitochondrial network. The luminally continuous mitochondria might represent electrically united systems [9].

Next we investigated whether in wild type cells, which contain a luminally discontinuous mitochondrial compartment, this discontinuity is stable, or whether an intermixing of the whole mitochondrial matrix occurs. In mitochondrial networks that were initially luminally discontinuous the distribution of the photoconverted GFP was subsequently followed by time-lapse microscopy (Fig. 5D–H; and Movie 2). In general we observed at latest after 10 min a complete labeling of the mitochondrial matrix with photoconverted GFP. Hence the frequent mitochondrial tubule fusion and fission events [2] ensure a complete intermixing of the mitochondrial matrix within a few minutes. In logarithmically growing budding yeast cells there is no stable segregation of the mitochondrial compartment within a single cell.

Subsequently we analyzed the causes for the observed discontinuities within the mitochondrial network. After photoconversion the luminally disconnected fragments were mostly those which were clearly separated from the main mitochondrial network. Besides we observed mitochondrial tubules, which were seemingly continuous (as judged by the green fluorescence) but luminally discontinuous (Fig. 5I–K). In principle these cases could represent inner membrane fission without concomitant fission of the outer membrane. But time-lapse microscopy demonstrates that in many instances such seemingly continuous tubules are in the process of division or fusion (Fig. 5I–L). Hence most, if not all, of these cases represent snapshots of ongoing tubule fissions or fusions. In wild type cells we did not obtain any evidence for a septation of the matrix (i.e. isolated matrix fragments enveloped by a common outer membrane), although very brief and transient septations would be indiscernible for our assay. Since no indications for the later supposition are available we conclude that in wild type budding yeast cells fission of the inner membrane is tightly linked to the fission of the outer membrane. To investigate whether in mutants restricted in outer membrane fission, inner and outer membrane fissions are still interweaved processes we analyzed the matrix continuity in mutants lacking Dnm1p and Fis1p.

3.6. *In cells lacking the mitochondrial fission components Dnm1p and Fis1p the continuity of the mitochondrial matrix is maintained*

Matrix targeted GFP in Δ*fis1* and Δ*dnm1* mutants was photoconverted in a small fraction of the mitochondrial network. Subsequently we imaged the distribution of the photoconverted GFP over the remaining network. In all analyzed cells (> 25 for each mutant) the photoconverted GFP spread over the whole mitochondrial network within less than 5 s (Fig. 6). We found the mitochondrial matrix of Δ*fis1* and Δ*dnm1* mutants to be continuous, although matrix constrictions were frequently observed (Fig. 6 and [2,26]). Hence matrix constrictions do not require the fission of a membrane. They appear to be independent of the outer membrane fission machinery.

Because Δ*fis1* and Δ*dnm1* mutants are deficient in outer membrane fission the continuity of the matrix indicates the concomitant lack of inner membrane fission. This finding also indicates that these processes are tightly interweaved in *S. cerevisiae*.

In contrast, *Caenorhabditis elegans* mutants which expressed dominant interfering mutant versions of DRP1, a homolog of yeast Dnm1p, harbored mitochondria with separated matrix compartments that were still connected by the outer membrane [27]. Hence it has been concluded that in this organism fission of inner and outer membranes can be uncoupled by eliminating functional DRP1. This might point

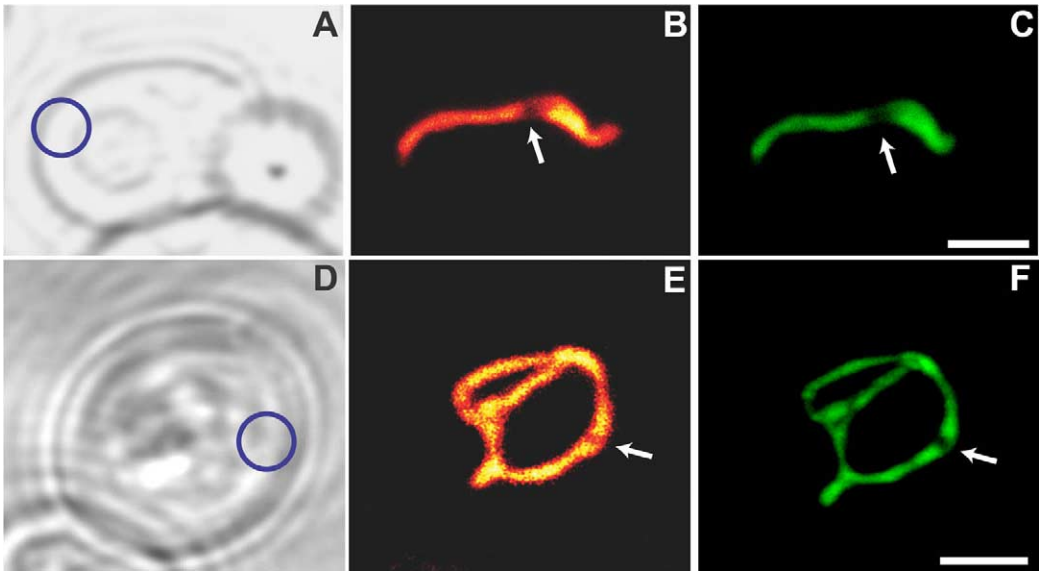

Fig. 6. Spot photoconversion of matrix targeted GFP expressed in Δ*fis1* and Δ*dnm1* mutants. A–C: GFP targeted to the matrix of Δ*fis1* mutants was photoconverted in a spatially confined area. A: Bright field image. The blue circle indicates the photoconverted area. B: Red fluorescence of the photoconverted GFP. C: Green fluorescence. D–F: Photoconversion of matrix targeted GFP in Δ*dnm1* mutants. D: Bright field image. E: Red fluorescence of photoconverted GFP. F: Green GFP fluorescence. Displayed are maximum intensity projections. Arrows indicate sites of matrix constrictions. Bars: 2 μm.

to important differences in the fission machineries of *C. elegans* and *S. cerevisiae*.

3.7. Conclusion

We have established GFP photoconversion as a method for investigating the luminal continuity of mitochondria in living budding yeast cells. Recently three promising fluorescent highlighters, PA-GFP [28], Kaede [20] and KFP1 [29] have been reported. Although each of these proteins has distinct advantages there are still drawbacks associated to them. Currently Kaede and KFP1 are problematic fusion tags because of their tendency to self-associate to form tetramers. PA-GFP and Kaede require expensive ultraviolet (UV) or two-photon optical equipment for activation. The major constraint of GFP photoconversion is its requirement for a low oxygen environment. However, for facultative anaerobes like budding yeast this environmental condition can be regarded as physiological. Due to its weak tendency for self-association GFP is an excellent tag for fusion proteins. Photoconversion requires relatively low light levels and can be effected in standard fluorescence microscopes. Using this technique we found that in budding yeast fission of outer and inner membranes appears to be tightly coupled. Matrix constriction and mitochondrial fission are accomplished by different processes. The data demonstrate that at a given point in time the highly dynamic mitochondrial compartment of *S. cerevisiae* generally consists of a single large luminally continuous part and occasionally some additional smaller fragments. In logarithmically growing cells a complete intermixing of the mitochondrial matrix occurs in less than 10 min.

Acknowledgements: We thank Marcus Dyba for help with the lifetime measurements and valuable discussions. We thank Dr. Benedikt Westermann, University Munich, for fruitful discussions. We are grateful to Prof. Dieter Gallwitz for allowing us to use his laboratory infrastructure. This work was partially supported by the Bundesministerium für Bildung und Forschung (BMBF) through grant 13N8353.

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