Improved affinity isolation strategy of metabolically aged OXPHOS complexes in budding yeast.

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Background

Mitochondrial proteins cannot be efficiently renewed in asymmetric cells such as neurons, where most mitochondria reside far from the nucleus — the origin of almost all mitochondrial protein transcripts. This results in the metabolic aging of OXPHOS subunits over time and is increasingly linked to mitochondrial dysfunction in neurodegenerative disease.

To study this process experimentally, our lab uses Recombination Induced Tag Exchange (RITE) in budding yeast (S. cerevisiae). Upon addition of β-estradiol, Cre recombinase is activated and excises a cassette sequence flanked by two loxP sites, switching the protein's epitope tag and creating separable 'old' (pre-switch) and 'new' (post-switch) protein populations that can be compared by mass spectrometry.

The existing RITE cassette uses a GFP tag (~26 kDa), which is large enough to risk disrupting OXPHOS complex function and assembly. Affinity isolation of aged complexes from total cell lysate has also been inefficient. This project addresses both limitations.

Approach

Improved RITE Cassette Design:
To reduce the size of the affinity tag, the coding sequence of the GFP tag in the RITE cassette was separated by an additional linker encoding for 3×FLAG tag and ERBV1 peptide bond skipping site (ref) (Figure 1). When translated, the fusion protein is broken into two leaving the 3xFLAG tagged protein and a separate GFP protein that reports the act of translation.

RITE Cassette Design

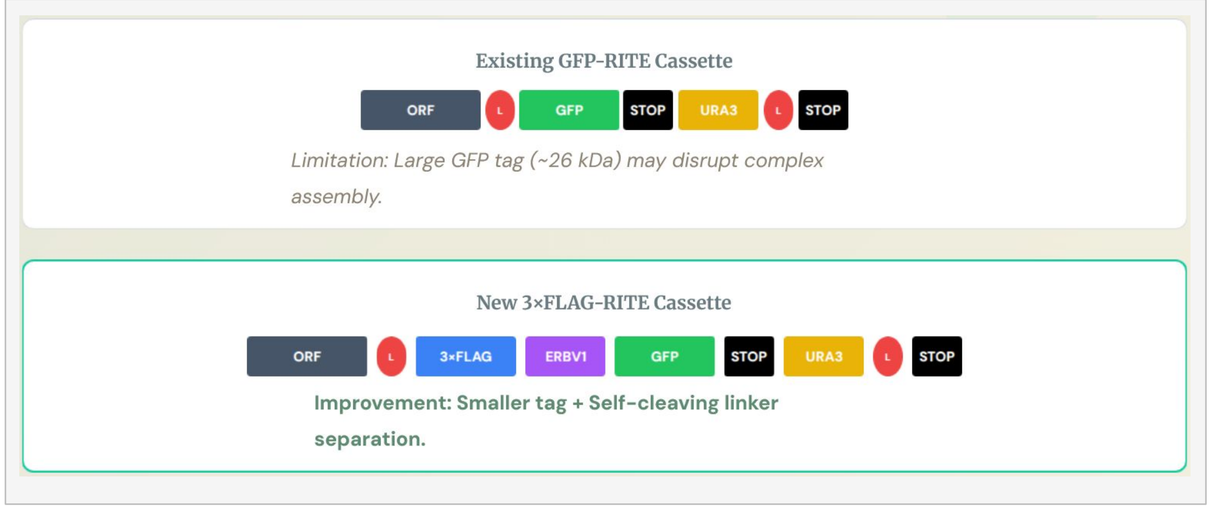
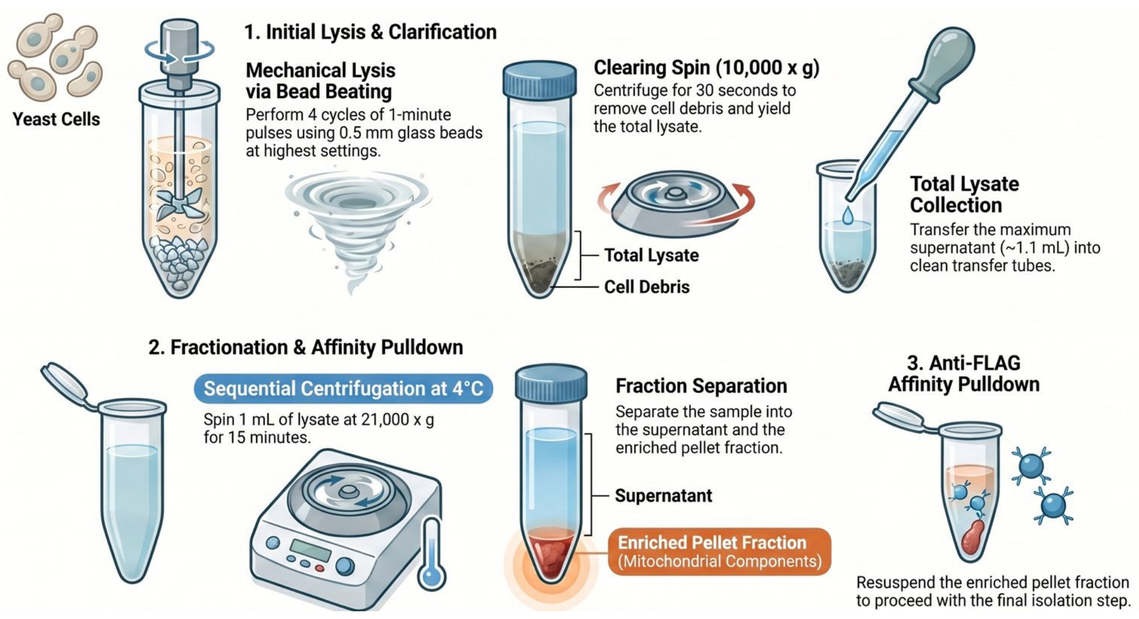


Fig. 1. Schematic of the existing GFP-RITE cassette (top) and the new 3×FLAG-RITE cassette (bottom). ERBV1 is a self-cleaving peptide sequence that separates the affinity tagged protein of interest and GFP that serves as reporter of tagged protein translation

Improved affinity isolation strategy of OXPHOS complexes

To improve the isolation of metabolically aged complexes cell lysates were additionally fractionated by centrifugation to isolate crude membrane fraction enriched in OXPHOS complexes.



Results — Expression of RITE tagged mitochondrial proteins analyzed by Fluorescence Microscopy

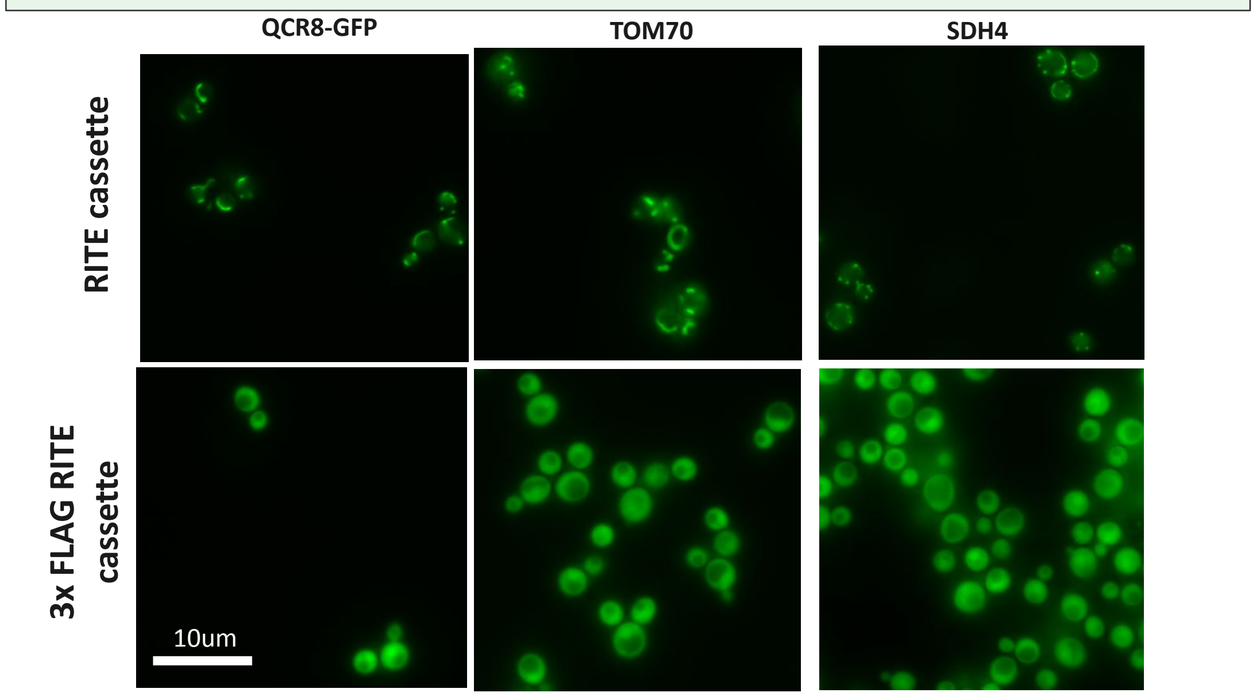


Fig. 2. Fluorescence microscopy analysis of three RITE-tagged yeast strains. Note that GFP reporter loses a characteristic tubular mitochondrial localization pattern in the case of 3xFLAG RITE cassette .

Results — Serial Dilution Growth Assay

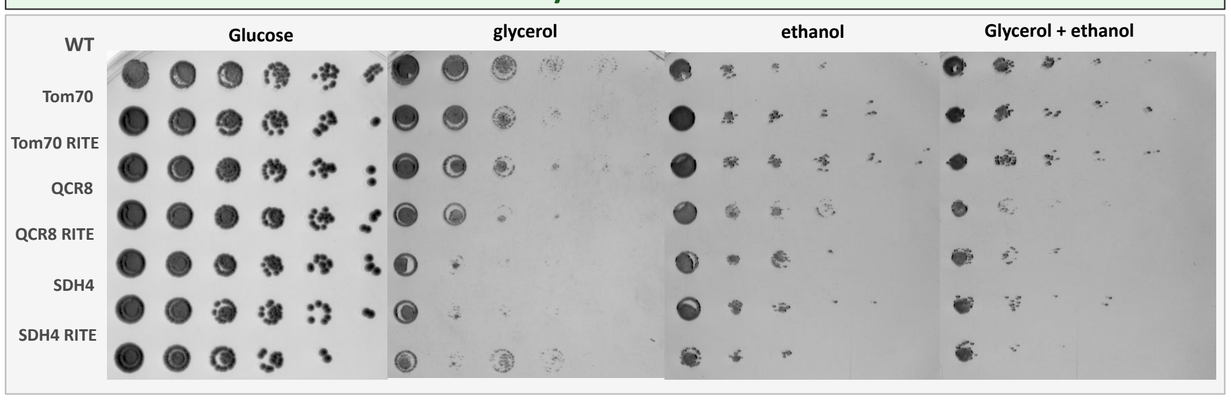


Fig. 3. Functionality of the RITE-tagged OXPHOS subunits analyzed by serial dilution growth assay in non-fermentable carbon sources. Strains plated in 5× serial dilutions on a fermentation medium (glucose) or non-fermentative media (glycerol, ethanol, ethanol + glycerol) to assess fitness impact of the tag on the OXPHOS function. Plates were imaged after 2 days of growth at 30C. Note that all strains grow comparably faster in the glucose medium. WT - unmodified control.

Results — Subcellular Fractionation & Enrichment

Subcellular fractionation by differential centrifugation was assessed as a pre-enrichment step before anti-FLAG affinity pulldown. Western blot compares GFP signal across lysate, supernatant and pellet fractions.

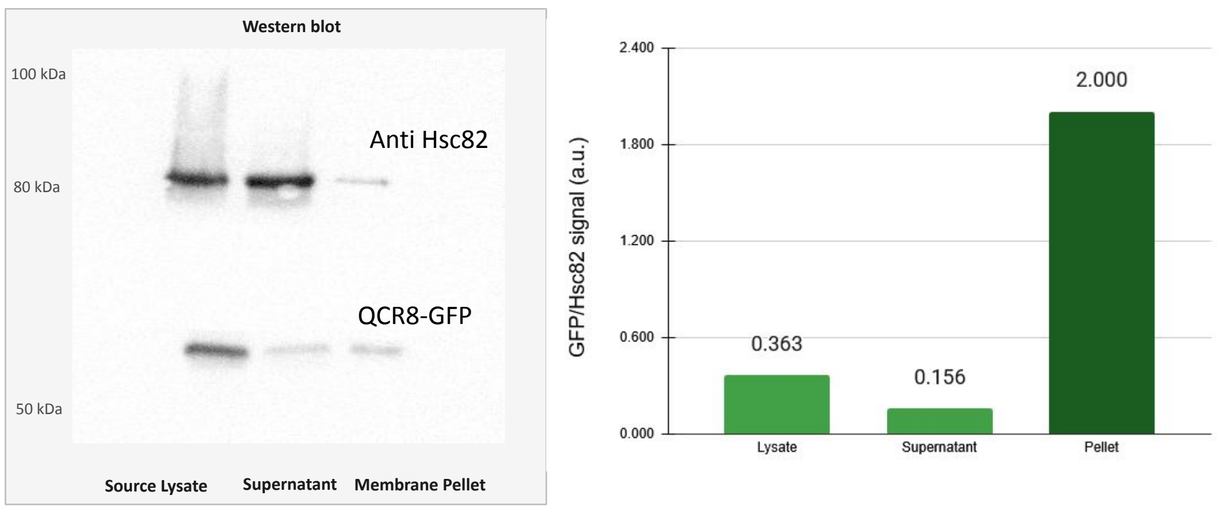


Fig. 4. Pre-enrichment efficiency of OXPHOS subunits by subcellular fractionation analyzed by Western blot-based quantification. Subcellular fractions of the yeast lysate (Source Lysate, Supernatant, Membrane Pellet) were probed for the relative content of GCR8-GFP and Hsc82 (serving as fiducial markers of OXPHOS and cytosolic protein content) by Western blotting. (Left) Representative image of the subcellular probed with anti-GFP (QCR8-GFP, ~51 kDa) and anti-Hsc82 (~80 kDa, loading control) antibodies. (Right) Quantitative analysis of the OXPHOS protein enrichment over cytosolic proteins using GFP band intensity normalized to Hsc82 as a readout. Note that the Membrane Pellet fraction shows greatest enrichment of the OXPHOS subunits.

Conclusions & Future Perspectives

Conclusions:

- A new 3×FLAG-RITE cassette was successfully constructed and validated by restriction digest and Sanger sequencing (clone 4)
- GFP reporter confirmed correct localization for QCR8, TOM70 and SDH4 by fluorescence microscopy
- Western blot quantification indicate that the pellet fraction enriches OXPHOS subunits relative to total lysate, supporting fractionation as an effective pre-enrichment step
- Serial dilution assays suggest a modest fitness cost of the OXPHOS subunit under respiration conditions.

Future Work:

- Confirm cassette integration at all five loci (QCR8, QCR9, SDH4, COX8, TOM70) by Pringle PCR
- Induce RITE switch with β-estradiol and validate tag switching efficiency by flow cytometry and western blot
- Perform anti-FLAG affinity pulldown from pellet fraction and assess co-purification of OXPHOS complex partners by silver stain / western blot
- Proceed to quantitative mass spectrometry (AP-MS) to identify chemical modifications in metabolically old vs new OXPHOS fractions

References

1. Swayne TC et al. (2011) Role for cER and Mmr1p in Inheritance of Mitochondria. Mol Cell 44(4):593–605.
2. Lindner AB et al. (2008) Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. PNAS 105(8):3076–3081.
3. Toyama EQ et al. (2016) Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. Science 351(6270):275–281.
4. Verzijlbergen KF et al. (2010) Recombination-induced tag exchange to track old and new proteins. PNAS 107(1):64–68.

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