Biosynthesis of the nitrogenase active-site cofactor precursor NifB-co in Saccharomyces cerevisiae

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The radical S-adenosylmethionine (SAM) enzyme NifB occupies a central and essential position in nitrogenase biogenesis. NifB catalyzes the formation of an [8Fe-9S-C] cluster, called NifB-co, which constitutes the core of the active-site cofactors for all 3 nitrogenase types. Here, we produce functional NifB in aerobically cultured Saccharomyces cerevisiae. Combinatorial pathway design was employed to construct 62 strains in which transcription units driving different expression levels of mitochondria-targeted nif genes (nifUSX and fdxN) were integrated into the chromosome. Two combinatorial libraries totaling 0.7 Mb were constructed: An expression library of 6 partial clusters, including nifUSX and fdxN, and a library consisting of 28 different nif genes mined from the Structure–Function Linkage Database and expressed at different levels according to a factorial design. We show that coexpression in yeast of the nitrogenase maturation proteins NifU, NifS, and FdxN from Azotobacter vinelandii with NifB from the archaea Methanocaldococcus jannaschii or Methanothermobacter thermautotrophicus thermotrophicus yields NifB proteins equipped with [Fe-S] clusters that, as purified, support in vitro formation of NifB-co. Proof of in vivo NifB-co formation was additionally obtained. NifX as purified from aerobically cultured S. cerevisiae coexpressing M. thermautotrophicus NifB with A. vinelandii NifU, NifS, and FdxN, and engineered yeast SAM synthase supported FeMo-co synthesis, indicative of NifX carrying in vivo-formed NifB-co. This study defines the minimal genetic determinants for the formation of the key precursor in the nitrogenase cofactor biosynthetic pathway in a eukaryotic organism.

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**Significance**

Nitrogen is a constituent of many essential biomolecules and plentiful on earth as inert N2 gas. For its assimilation by eukaryotes, N2 must be converted to a metabolically tractable form such as ammonium. Such conversion is catalyzed by nitrogenase, an enzyme produced by a select group of microorganisms called diazotrophs. Crop yields necessary to feed the world’s population have critically depended on applying nitrogenous fertilizers. Incorporation of prokaryotic determinates required to produce active nitrogenase into crop plants would have enormous economic and environmental benefits. The active-site cofactors of all nitrogenases have a common metallocluster precursor synthesized by NifB. Here, we identify the genetic determinants for NifB function in mitochondria of Saccharomyces cerevisiae, thereby advancing prospects to generate N2-fixing crops.
the same domain architecture. Most variants contain either a standalone SAM domain or a SAM domain together with a carboxyl (C)-terminal NifX-like domain (e.g., A. vinelandii NifB) (18). A His-tagged single-domain NifB variant from the thermophilic archaeon *Methanocaldococcus infernus* produced in yeast could be partly solubilized upon heat treatment and purified using metal-affinity chromatography. This as-isolated form of NifB was loaded with ~3 Fe atoms per monomer (17), similar to the same form of NifB produced by and purified from *Escherichia coli* cells (12). These experimental results contrast with the 12 Fe atoms expected for a fully loaded NifB protein harboring 3 [4Fe-4S] clusters (11). In this case, yeast-expressed His-tagged NifB was only active following [Fe-S] cluster re-constitution, and, therefore, the exact genetic requirements for producing a functional NifB in yeast could not be determined.

Targeting all required gene products to mitochondria represents a challenge as the various N2-fixing systems are very complex from both genetic and biochemical perspectives (19, 20). Recent work has resolved possible ways that separately, or in combination, provide opportunities to simplify this problem. One of these involves functional replacement of certain required prokaryotic components by proteins of plant origin (21, 22), and another involves fusion of certain *nif* genes to produce polyproteins amenable to posttranslational processing to yield individually active components (23, 24). Transfer of the minimal suite of genes required to produce active Mo-dependent nitrogenase in a model prokaryotic system to the chromosomes of *S. cerevisiae* has been reported. In this case, expression levels and targeting approaches were balanced by designing combinatorial libraries comprising 9 *nif* genes of the *A. vinelandii* Mo-nitrogenase (*nifHDKUSMBEN*) (25). Ultimately, expression and mitochondria targeting were observed for all 9 gene products, and the NifDK tetramer was correctly assembled. However, biochemical characterization revealed that NifDK produced in this system lacked FeMo-co, resulting in accumulation of inactive and unstable apo-NifDK. Thus, a mitochondrial assembly remains a major barrier for the generation of a N2-fixing eukaryote.

Here, we apply combinatorial pathway design and assembly to investigate the function of 28 selected *nif* gene products in aerobically cultured yeast. Through iterative testing involving 62 NifB pathway variants, we found that NiB from *M. infernus* and *Methanothermobacter thermotaotrophicus* can be produced in yeast. In both cases, coexpression of *A. vinelandii* NifU, NifS, and FdxN synthesized NiB that, as purified, supported FeMo-co formation in vitro. Importantly, one *M. thermautotrophicus* NiB pathway generated NiX that contained in vivo-formed NiB-co.

**Results**

**Library Strategy and Design.** The starting hypothesis was that genetic factors influencing NiB activity would include expression levels of NiB and the accessory proteins NifU, NifS, NifX, and FdxN, as well as intrinsic properties encoded in distinct NiB variants. Different combinations of these factors were tested by applying pathway library design (25, 26), which enabled combinatorial construction of strains containing specific selection of genes, regulatory sequences, targeting signals, and purification tags (Materials and Methods).

First, we designed a library of 6 parental strains to optimize stable expression of *A. vinelandii* nifU, nifS, nifX, and fdxN genes postulated as necessary to determine NiB activity in yeast. For nifU and nifS, expression was controlled by promoter/terminator combinations previously used (25), whereas fdxN and nifX expression was newly designed with the constraint that NifX should be equal or up to 36-fold higher than FdxN (SI Appendix, Fig. S1 A and B and Tables S1 and S2). The rationale was that NiX expression levels have been reported to be generally higher than other Nif proteins involved in FeMo-co synthesis (27) and that NiX can stabilize produced NiB-co (8). FdxN carried a C-terminal HA-tag to facilitate immunodetection (Fig. 2A), while NiX was fused to the C-terminal end of glutathione S-transferase (GST) to facilitate purification. All proteins were targeted to the mitochondria of *S. cerevisiae* using sequence variants of the SU9 signal selected to limit undesired homologous recombination (Fig. 2A and SI Appendix, Supplementary Text sequences). Parental strain 3 produced the most consistent protein expression and was used as foundation for the second library (SI Appendix, Fig. S1 C and D).

NiB proteins are notoriously difficult to study due to instability (11–13, 28). To find NiB proteins better suited for expression in eukaryotic cells, we mined the Structure–Function Linkage Database (29) for 28 different *nif* gene sequences and synthesized them with codon optimization for expression in *S. cerevisiae*. Because the ultimate goal is to engineer nitrogenase in plants, *nif* genes were selected according to the following criteria: 1) those originating from or being associated to photosynthetic organisms, 2) those originating from organisms with aerobic lifestyle, and 3) those whose products have been previously shown to support NiB-co formation (Fig. 2B, SI Appendix, Table S3, and Dataset S1). As it was previously shown that somewhat-soluble NiB protein from the archaeon *M. infernus* could be produced in *S. cerevisiae* (17), the selection included additional archaeal *nif* genes. Thus, 25 *nif* genes were of bacterial origin (13 Proteobacteria, 2 Cyanobacteria, 2 Firmicutes, 1 Chlorobi, and 1 Chloroflexi) and 3 of archaeal origin. Eighteen harbored the C-terminal NiX-like domain present in NiB proteins with a 2-domain structure, while the remaining 10 candidates contained only the conserved SAM-radical domain (SI Appendix, Table S3 and Dataset S1) (18).

A library of 56 yeast strains was built by factorial design in which each *nif* was tested at 2 expression levels predicted to differ by 9-fold (Fig. 2 C and D and SI Appendix, Fig. S1A and Table S2), together with *nifU*, *nifS*, *fdxN*, and *nifX* as expressed in parental strain 3 (Fig. 2A and SI Appendix, Table S1). To facilitate NiB detection and isolation, a purification tag was placed between the amino (N)-terminal SU9 mitochondria targeting signal and the NiB-encoding region (Fig. 2D and SI Appendix, Supplementary Text sequences). Three different tags (10x His, 1x Strep, and 2x Strep) were tested for their effect on expression and solubility of NiB from *M. infernus*, used as reference protein (SI Appendix, Fig. S2, Table S4, and Supplementary Text sequences). Because the solubility of the Twin-Strep (TS)-tagged NiB protein was highest (SI Appendix, Fig. S3), and because Strep-Tactin–based purification procedures do not rely on metal-affinity resins that can remove the labile [4Fe-4S] clusters carried by NiB proteins, the TS-tag was chosen for the
Fig. 2. Generation of yeast NifB library. (A) S. cerevisiae CEN.PK113–7D was integrated with nifS, nifU, nifX (GST-NifX), and FdxN (FdxN-HA) at varying expression levels to create 6 parental strains (P5) for NifB expression. (B) Phylogenetic distribution of nifB genes selected for the library. (C) The genetic schematic of the nif constructs and their location in the S. cerevisiae genome are shown. Promoters and terminators for FdxN, NifX, and NifB that were utilized in different combinations within the library are separated by “||” symbols. The cluster was inserted in the direction of leading-strand replication, between 459,247 and 458,821 bp on chromosome XV in S. cerevisiae CEN.PK113–7D. Part sequences are provided in SI Appendix. (D) Fifty-six strains were constructed to express 28 nifB genes at 2 levels in the parental strain PS3. Promoters and terminators for high (P26-T22) and medium (P17-T22) nifB expression were chosen from previous designs (25). Each line represents an assembly step (see Materials and Method and SI Appendix for further details). (E) Western blot analysis of soluble protein extracts of wild-type S. cerevisiae (WT) and S. cerevisiae strains expressing the 6 soluble NifB candidates as indicated. Arrows indicate migration of full-length NifB proteins. Star indicates nonspecific signal. Ponceau-stained membrane is shown in SI Appendix, Fig. S5C. (F) Workflow followed in this report. Six parental yeast strains were generated to tune expression of A. vinelandii nifS, nifU, fdxN, and nifX (I) (A and SI Appendix, Fig. S1). Twenty-eight nifB genes at 2 different expression levels were integrated with accessory genes as in parental strain 3 (PS3), generating 56 yeast strains (II) (B–D). Sixteen distinct NifB proteins could be detected in yeast protein extracts (III) (SI Appendix, Fig. S5A), of which 6 accumulated as soluble proteins (IV) (E). These nifB candidate genes were transferred to high-expression vectors, resulting in 5 mainly intact full-length NifB proteins (V) (SI Appendix, Fig. S9). Two of the candidates could be purified at high yields (V) (SI Appendix, Fig. S10), where the M. infernus NifB accumulated with higher Fe-S cluster occupancy (VII) (SI Appendix, Fig. S11) and was selected for analysis of activity, Fe-S clusters, and accessory gene dependency (VIII) (Figs. 3 and 4 and SI Appendix, Figs. S14–S18). In vivo NifB activity was shown by isolation of a NifX/NifB-co complex in a S. cerevisiae strain expressing the M. thermautotrophicus NifB protein (IV) (Fig. 5 and SI Appendix, Fig. S24). A summary of the screen is shown in SI Appendix, Table S3.
library screen (Fig. 2D and SI Appendix, Supplementary Text sequences). The enhanced solubility of M. infernus TS-NifB at moderate temperature was unexpected as purification of a C-terminally His-tagged version required prior heat treatment (17). Temperature-independent solubility of this engineered variant was confirmed (SI Appendix, Fig. S4 and Table S4), suggesting that addition of a neutral N-terminal extension (such as TS) could prevent its aggregation and/or membrane sequestration.

Sixteen distinct nifB genes generated polypeptides with migration in sodium dodecyl sulfate (SDS) gels that corresponded to those expected after mitochondrial import and SU9 processing (SI Appendix, Table S3 and Fig. S5 B and C and Fig. 2E) and were selected for further analysis. The workflow of this study is summarized in Fig. 2F.

Purification of NifB Candidates Expressed in Aerobically Growing Yeast. The 6 soluble NifB candidates were screened for activity using previously described NifB-dependent in vitro FeMo-co synthesis and insertion assay (5). This assay relies on the activation of apo-NifDK present in crude extracts of a NifB-deficient A. vinelandii strain (6, 30). Soluble extracts from aerobic flask-cultured S. cerevisiae strains were subject to anaerobic small-scale Strep-Tactin pull-downs to enrich NifB, which was then tested for NifB activity. No apo-NifDK activation was detected in any of the samples (SI Appendix, Fig. S6 A and B), and Western blot analysis showed that NifB levels were insufficient to support this screening assay (SI Appendix, Fig. S6C). Sufficient biomass was obtained from aerobically grown fermenter cultures of strain SB187Y expressing M. infernus NifB, which permitted the purification of approximately 3 mg of NifB (SI Appendix, Fig. S7 A–C). As-isolated NifB lacked activity, as previously reported (SI Appendix, Fig. S7D). It was observed that FdxN accumulation in fermented cells decreased over time and that GST-NiFX suffered degradation, which precluded NiFX purification (SI Appendix, Fig. S7E). Differential expression of NiFB and FdxN could be problematic as FdxN is important for in vivo NifB activity (15). The protein extracts were also prone to protein precipitation, which hindered further scale-up of the purification procedure as well as interpretation of the results.

Although NifB is not highly expressed under diazotrophic conditions in their natural hosts (27), in-depth biophysical characterization of the protein and its clusters require large amounts of pure protein. To investigate the effect of NiFU, NiFS, and FdxN on NiFB [Fe-S] cluster content and activity, we transferred the genes encoding the 6 soluble NifB candidates identified in the library screening to yeast expression vectors (SI Appendix, Fig. S8, Table S4, and Supplementary Text sequences). The nifX gene was not included at this stage, because NiFX is not required for NifB function and, in addition, it could trigger release of NiFB from NiFU. Expression of all genes was controlled by GAL regulatory elements previously used for effective production of NiFU, NiFS, FdxN, and NiFB (17). All NiFB candidates accumulated at high levels, except for the Synchococcus sp. JA-3-3Aβ variant (SI Appendix, Fig. S9). NiFB from Methanosarcina acetivorans produced significant levels of a faster migrating isomorph indicating protein degradation, so this construct was excluded from further analyses. Aerobic fermenter cultures for the remaining 5 strains were prepared, and NiFB proteins were purified. Only NiFB from M. thermautotrophicus and M. infernus yielded mainly soluble protein when produced at the high levels required for their biochemical characterization (SI Appendix, Fig. S10). Notably, both M. thermautotrophicus and M. infernus are archaea, and their NiFB proteins lack the C-terminal NiFX-like domain (SI Appendix, Table S3). Ultraviolet (UV)-visible spectra indicated that both proteins accommodated [Fe-S] clusters (SI Appendix, Fig. S11). Because the cluster occupancy appeared higher in the M. infernus NiFB and given previous experience with this analysis, this variant was selected for in-depth biophysical analysis and for testing the requirement of NiFU, NiFS, and FdxN for NiFB function.

Biophysical Properties of M. infernus NiFB. For clarity, mitochondria-targeted TS-NifB produced in yeast and in the absence of any other prokaryotic component is hereafter denoted as NifBUSF. The NiFB species produced in yeast in combination with mitochondrial NiFU, NiFS, and FdxN are denoted by a superscript of the corresponding coexpressed nif gene product. For example, the NiFB species produced in combination with NiFU, NiFS, and FdxN is indicated as NiFBUSF (SI Appendix, Fig. S12 and Table S4). Because M. infernus NiFBUSF accumulated as a largely soluble protein, it could be purified as a dark brown protein (Fig. 3A–D). The influence of coexpressing NiFU, NiFS, and FdxN separately, or in combination, on NiFB capacity for SAM-dependent NiFB-co formation could also be evaluated. Proper protein targeting to yeast mitochondria and subsequent processing was confirmed by migration of the corresponding proteins on SDS–polyacrylamide gel electrophoresis (SDS/PAGE) and, in the case of NiFB, by N-terminal amino acid sequencing (Fig. 3M and SI Appendix, Fig. S13). Various NiFB species anisotropically isolated from aerated fermenter cultures of yeast cells yielded about 6 mg of protein per 100 g of cells, and the metal content and UV-visible spectra of these species were determined (SI Appendix, Figs. S14 and S15 and Table S5).

NiFB is known to carry 3 distinct [4Fe-4S] clusters (12). One of these, designated RS, is associated with SAM and is a permanent catalytic cluster, whereas the other 2 are accessory clusters, designated AC1 and AC2, which are ultimately fused to form NiFB-co. Because the UV-visible spectrum and Fe concentration indicated that the M. infernus NiFBUSF and NiFBUS species could have the highest cluster occupancy (Fig. 3C and SI Appendix, Fig. S15 and Table S5), it was of interest to examine the electron paramagnetic resonance (EPR) spectra of these proteins. The EPR spectrum of as-isolated M. infernus NiFBUSF measured at 12 K exhibited S = 1/2 signals typical of reduced [4Fe-4S] clusters (Fig. 3E and SI Appendix, Fig. S16). This assignment was confirmed by temperature-dependent measurements, which showed signal disappearance above 70 K (SI Appendix, Fig. S17) (31). The signal was well reproduced by including 3 different [4Fe-4S] clusters in spectral simulations (Fig. 3E), having g values almost identical to those of the clusters found in the reconstituted M. infernus NiFB expressed in E. coli (12). Total spin concentration of the S = 1/2 [4Fe-4S] signals was ∼1.5 mM, which translated to ∼6 mM Fe. The Fe concentration estimated by EPR was also in good agreement with chemical Fe quantitation (∼7 mM), indicating that almost all Fe in NiFBUSF was in the form of [4Fe-4S] clusters. In contrast, only one type of [FeS]4 clusters (AC1 cluster; ref. 12) was observed in the EPR spectrum of the as-isolated NiFBUS proteins (SI Appendix, Fig. S18), while the RS and AC2 clusters were missing.

Purification and Activities of M. infernus and M. thermautotrophicus NifB Species Produced in Yeast. The abilities of various NiFB species to support FeMo-co synthesis was tested using the above-described in vitro FeMo-co synthesis and insertion assay (5). As-isolated M. infernus NiFBB had no ability to support in vitro FeMo-co synthesis, and NiFBB and NiFBUS had only minimal capacity relative to NiFBUSF (Fig. 4A). In vitro FeMo-co synthesis performed in reactions containing only purified NiF components (SI Appendix, Fig. S19) confirmed that as-isolated M. infernus NiFBUSF could support FeMo-co formation without requiring prior [Fe-S] cluster reconstitution. A dose-dependent increase in apo-NifDK activation was observed in this system when NiFBUSF was used as the limiting component in activation (Fig. 4B). These experiments established that NiFBUSF can provide NiFB-co to support FeMo-co formation in the in vitro system. However, they did not establish whether NiFB-co was already present within the as-isolated NiFBUSF, or if SAM was required to convert a fraction of [4Fe-4S] clusters contained
within as-isolated NiFBUSF to NiFB-co. This question was resolved by showing that NiFB-co formation and apo-NiFDK activation required both NiFBUSF and SAM (Fig. 4C and SI Appendix, Fig. S20), demonstrating that preformed NiFB-co was not contained within as-isolated NiFB.

*M. thermautotrophicus* NiFBUSF was also purified with [Fe–S] clusters and supported in vitro synthesis of FeMo-co and apo-NiFDK activation (Fig. 4D–G and SI Appendix, Fig. S21). *M. thermautotrophicus* NiFBUSF purifications had higher yields than those of *M. infernus* NiFBUSF (average, 17.9 mg of NiFBUSF per 100 g of cells; n = 2), while the Fe content was slightly lower (average, 4 Fe atoms per NiFBUSF monomer; n = 2) (SI Appendix, Table S5).

**Isolation of NiFX with Bound FeMo-co Precursor Produced in Yeast.** Both *M. thermautotrophicus* and *M. infernus* NiFB proteins lack the C-terminal NiFX-like domain, and it is possible that they are incapable of accumulating NiFB-co. As NiFX has demonstrated affinity for NiFB-co, it was built into the pathways to trap any in vivo formed NiFB-co. To overcome the instability of GST-NiFX observed in the library screening, GST was replaced by a TS-tag–tobacco etch virus (TEV) site cassette (SI Appendix, Fig. S22 and Supplementary Text sequences). In addition, as accumulation of *M. thermautotrophicus* and *M. infernus* NiFB proteins in yeast was very high, endogenous mitochondria SAM levels could be limiting. A mitochondria-targeted variant of the cytosolic SAM synthase (Sam1p) was therefore engineered following the strategy used by Marobbio et al. (32) (SI Appendix, Fig. S22 and Supplementary Text sequences). This approach is similar to the overexpression in *E. coli* of the SAM synthase gene metK used to obtain functional *M. infernus* NiFB (12). Functionality of mitochondria-targeted Sam1p was verified by growth of *sam5Δ* cells transformed with SU9-Sam1p-FLAG in yeast extract–peptone media containing a nonfermentable carbon source (32) (SI Appendix, Fig. S23).

NiFB and NiFX proteins were purified simultaneously from mitochondria of *S. cerevisiae* strains additionally coexpressing NiFU, NiIS, FdxN, and Sam1p (Fig. 5A and B and SI Appendix, Fig. S24). Both *M. infernus* NiFBUSF-SAM and *M. thermautotrophicus* NiFBUSF-SAM supported SAM-dependent FeMo-co formation and apo-NiFDK reconstitution using purified protein components (Fig. 5C and D). As NiFX does not possess catalytic activity but acts as carrier of NiFB-co, synthesis of FeMo-co and

![Image 1](https://example.com/image1)

![Image 2](https://example.com/image2)

![Image 3](https://example.com/image3)

![Image 4](https://example.com/image4)

**Fig. 3.** Expression and purification of *M. infernus* NiFB carrying [Fe–S] clusters from yeast. (A) Immunoblot analysis of protein expression in total extracts of wild-type *S. cerevisiae* (WT) and *S. cerevisiae* strains used for NiFB purifications (strain SB30Y, for expression of NiFB*; SB31Y, for expression of NiFBUSF; SB32Y, for expression of NiFBUSF; SB33Y, for expression of NiFBUSF-SAM, (B and C) Coomassie staining (B) and as-isolated and air-exposed UV-visible spectra (C) of NiFBUSF protein. (D) Appearance of NiFBUSF (purification 13; SI Appendix, Table S5) obtained from 315 g of yeast cells following biotin-elution and desalting (total volume, about 13 mL). (E) X-band EPR spectra of NiFBUSF (purification 14; SI Appendix, Table S5) (1) and subcomponents of spectral simulation for NiFBUSF (2 to 4). Experimental data are shown in black solid lines, while overall spectral simulations are shown in red dotted lines. The g values of each species, spin concentration of the subcomponents, and cluster nomenclature (adapted from ref. 12) are indicated in the figure.

![Image 5](https://example.com/image5)

**Fig. 4.** Genetic and biochemical requirements for NiFB functionality. (A) In vitro synthesis of FeMo-co and apo-NiFDK reconstitution assay using ΔnifB A. vinelandii (UV140) CFEs and 12.5 μM as-isolated *M. infernus* NiFB* (purple), NiFBUSF (green), NiFB (blue), or NiFBUSF (red). Activity is represented as nanomoles of ethylene produced per minute and assay (left y axis). Error bars represent means ± SD (n = 3; NiFB*, NiFB, and NiFBUSF; n = 4; NiFBUSF). The shapes of symbols for each yeast strain indicate whether *M. infernus* NiFB was purified from cells originating from the same or different fermenters. Average Fe content of each NiFB is indicated with a black thick line (right y axis; SI Appendix, Table S5). (B) Titration of in vitro FeMo-co synthesis and apo-NiFDK reconstitution using purified proteins (see Materials and Methods for details) and as-isolated *M. infernus* NiFBUSF (purification 13; SI Appendix, Table S5). Activity is represented as nanomoles of ethylene produced per minute and milligram of NiFDK. Error bars represent means ± SD (n = 2). Specific activities of holo-NiFDK and NiFB-codependent activated apo-NiFDK determined under the same reaction conditions were, respectively, 1,314 and 334 nmol of ethylene formed per minute and milligram of NiFDK protein. (C) Requirement of SAM for *M. infernus* NiFBUSF-dependent in vitro FeMo-co synthesis and apo-NiFDK reconstitution in a completely defined assay; 5 μM *M. infernus* NiFBUSF was used per assay (purification 13; SI Appendix, Table S5). Activities are compared to complete conditions (see Materials and Methods for details) and reported as nanomoles of ethylene produced per minute and milligram of NiFDK protein. Error bars represent means ± SD (n = 2). Specific activities of holo-NiFDK and NiFB-codependent activated apo-NiFDK determined under the same reaction conditions were, respectively, 1,314 and 334 nmol of ethylene formed per minute and milligram of NiFDK protein. (D) As-isolated and air-exposed UV-visible spectra of *M. thermautotrophicus* NiFBUSF protein. (E and F) Appearance (E) and Coomassie staining (F) of *M. thermautotrophicus* NiFBUSF obtained from 55 g of yeast cells following biotin-elution, desalting, and concentration. (G) *M. thermautotrophicus* NiFBUSF-dependent in vitro FeMo-co synthesis and apo-NiFDK reconstitution using purified proteins (see Materials and Methods for details); 5 μM as-isolated *M. thermautotrophicus* NiFBUSF was used in the assay. Error bars represent means ± SD (n = 2). Specific activities of holo-NiFDK and NiFB-codependent activated apo-NiFDK determined under the same reaction conditions were, respectively, 1,314 and 334 nmol of ethylene formed per minute and milligram of NiFDK protein.
that mitochondria provide a suitable environment for production of the O₂-sensitive nitrogenase proteins (16). The study also showed that activation of mitochondrial-targeted NifH only required additional coexpression of its associated maturase NifM. Thus, endogenous yeast mitochondrial [Fe-S] cluster biosynthetic machinery sufficed to provide NifH with its essential [4Fe-4S] cluster, which is normally provided by NifU and Nifs (33). This result suggested that not all nif-gene products essential for functional assembly of an active nitrogenase in a model prokaryotic system would necessarily be required for assembly of an active nitrogenase in a particular eukaryotic system. In other words, certain essential prokaryotic components can be replaced by eukaryotic proteins having similar functions. However, as discussed below, the present work reveals that this conclusion is not necessarily correct in the case of NifU and Nifs, as they are required for formation of active NifB. Following the concept of system simplification and gene transfer reduction, a synthetic biology approach has been applied to establish that ferredoxin-NADPH oxidoreductases and ferredoxins of plant organelle origin can serve as electron sources to support nitrogenase catalysis (21). In another study, synthetic gene fusions and posttranslational processing enabled the regrouping of the 14 *Klebsiella oxytoca* genes required for heterologous expression of an active nitrogenase in *E. coli* into only 5 “giant” genes (23). Both strategies are excellent examples of how synthetic biology can be employed to simplify the challenge of endowing eukaryotic organisms with the capacity to reduce N₂ (22, 24).

Taking into consideration recent progress in overcoming the anticipated problem of O₂ sensitivity of nitrogenase components by targeting them to mitochondria, as well as the potential of using synthetic biology to reduce the genetic complexity of the system, in vivo formation of the nitrogenase active-site cofactor remains as one of the most significant obstacles to producing an active nitrogenase in eukaryotes. Given that NifB-co is a common precursor required for assembly of the corresponding active-site cofactors contained in all 3 nitrogenase types, formation of active NifB is critical to endowing any eukaryote with the capacity for N₂ reduction. By employing synthetic biology to simultaneously test multiple factors influencing NifB function, the present work shows that a modified archaeal NifB variant expressed in aerobic yeast cultures, in combination with NifU, NifS, and FdxN accessory proteins from a diazotrophic Proteobacterium, is active without a requirement further in vitro reconstitution. Expression and solubility levels of the different NifB variants tested here could not be anticipated. For example, although the NifB originating from *Gloeothece sp. (KO68DGA)* and *Cyanothece sp. (ATCC 51142)* showed more than 95% identity, NifB accumulation was only detected for the *Gloeothece sp. (KO68DGA)* protein. Notably, some NifB variants were detected only when expressed at high levels, while others worked only at low levels. Moreover, it was not known a priori that for accessory genes, some combinations of expression levels would result in complete abrogation of NifB expression. In addition, a surprisingly small number of NifB proteins were soluble. Although both *M. thermautotrophicus* and *M. infernus* NifB proteins were predicted to be stable (Dataset S1), expression of other NifB sequences with similar low instability index could not be detected, highlighting that NifB accumulation was not easily anticipated from sequence information or from prior observations. One important observation is that both nifB genes were sourced from extreme thermophiles, implying that such proteins may be more stable in this heterologous expression system. Overall, because a library-based approach revealed these kinds of dependencies, we anticipate that in the future, methods to facilitate simultaneous testing of multiple design factors will be important for the engineering of pathways of increasing complexity.

A requirement for NifU and Nifs to produce active NifB was not expected because the maturation of [4Fe-4S]-containing NifH in yeast does not require NifU and Nifs. Namely, for the assembly and delivery of the [4Fe-4S] cluster contained in yeast-expressed apo-NifDK reconstitution using Nix/NifB-co complex does not require SAM. We therefore performed SAM-independent FeMo-co synthesis assays using NifX purified from yeast expressing *M. infernus* NifB<sub>USF-SAM</sub> or *M. thermautotrophicus* NifB<sub>USF-SAM</sub>. No substantial NifDK activity was measured with NifX purified from yeast expressing *M. infernus* NifB<sub>USF-SAM</sub> (Fig. 5E). On the contrary, NifX purified from yeast expressing *M. thermautotrophicus* NifB<sub>USF-SAM</sub> generated significant NifDK reconstitution (Fig. 5F), implying that NifX preparations contained bound NifB-co and that mitochondrial *M. thermautotrophicus* NifB<sub>USF-SAM</sub> was active in vivo. Some NifB<sub>USF-SAM</sub> contamination was observed in purified NifX fractions (Fig. 5B), but its role in SAM-independent FeMo-co synthesis was ruled out by demonstrated lack of activity of pure *M. thermautotrophicus* NifB<sub>USF-SAM</sub> in the in vitro FeMo-co synthesis assay (Fig. 5G).

**Discussion**

The first report of successful expression of functional NifH (dinitrogenase reductase) in aerobically grown *S. cerevisiae* established...
NifH, it appears that the function of NifU and NifS is suppressed by the yeast-encoded scaffold (ISU) and cysteine desulfurase (NFS1). Why this is not the case for NifB [Fe-4S] clusters could be related to the complexity of this enzyme containing 3 different [Fe-4S] clusters compared to a single [Fe-4S] in NifH.

It had been shown that a ΔnifU mutation in Klebsiella pneumoniae decreased NifB levels by 10-fold and abolished NifB-co activity in extracts (13). By uncoupling gst-nifB expression from nif regulation, similar amounts of GST-NifB accumulated independently of the nifUS genetic background. Furthermore, GST-NifB produced by this method was replete with Fe-S species and could be isolated with bound NifB-co. Both GST-NifB variants (expressed in presence or absence of nifUS) could activate apo-NifDK to similar levels, although overall NifB-co levels were much lower in extracts of the ΔnifU strain. This result contrasts with our results in S. cerevisiae, where NifU and NifS are required to produce NifB protein with high [Fe-S] cluster occupancy and no bound NifB-co was found. It should be noted that while NifB was produced in K. pneumoniae under strict anaerobic conditions, the S. cerevisiae strains used here were cultured under aerobic conditions. In addition, the different tags used for protein purification can influence metal content and activity, and the properties of the isolated proteins may not necessarily reflect the characteristics of the native protein. This could, for example, explain the different amounts of NifX-bound NifB-co in S. cerevisiae strains carrying nifB from M. infernus or M. thermotrophicus.

The involvement of FdxN in NifB maturation might also be relevant to understand the function of NifU and NifS. The U-type [Fe-S] proteins, of which NifU was the first member to be discovered (34), provide cysteine desulfurase-dependent scaffolds for the assembly of simple [Fe-S] clusters destined for maturation of other [Fe-S] cluster-containing proteins (33). In some cases, the U-type scaffold is not the direct donor to a particular client [Fe-S] protein but, instead, an intermediate carrier is involved. Although FdxN is annotated as a ferredoxin, its apparent involvement in supporting the acquisition of [Fe-S] clusters by NifB, rather than in SAM-dependent formation of NifB-co, could indicate a role as intermediate [Fe-4S] carrier rather than as electron donor. In some cases, primary U-type scaffolds can function in the absence of the intermediate carrier, but only at a low level, and this could explain the very low level of NifB-co in NifS+ΔnifU strains (13). This suggests that NifU and NifS could be essential to produce active NifH in S. cerevisiae, such as NifU and NifS, are necessary to produce active NifB; and 5) complementary assembly factors produced by highly divergent bacteria can be combined and sorted to achieve the production of building blocks essential to formation of an active nitrogenase in eukaryotes.

Materials and Methods

Strains, Media, and Molecular Biology for Generation of Yeast Libraries. S. cerevisiae CEN.PK113-7D (MATa URA3 TRPI LEU2 HIS3 MAL2–8c SUC2) was the host strain for all library constructs and grown at 30 °C in yeast extract–peptone–dextrose media, with 200 μg/mL G418 added when appropriate. Yeast transformations were carried out according to the lithium acetate method (35, 36). Chemically competent E. coli DH5α (New England Biolabs) was used as a cloning strain and grown at 37 °C in lysogeny broth media with appropriate antibiotics (100 μg/mL carbenicillin or 25 μg/mL kanamycin) and inducer (100 μM of 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was spread and dried on plates for blue/white-screening when appropriate.

All Sanger sequencing reactions were performed by Quintara Biosciences.

Plasmid isolations were performed with Qiagen Qiaprep kits. Genomic DNA was isolated using the Promega Wizard Genomic DNA Preparation Kit. Gel electrophoresis was carried out using 1% agarose Gels according to the lithium acetate method (35, 36). Chemically competent E. coli DH5α (New England Biolabs) was used as a cloning strain and grown at 37 °C in lysogeny broth media with appropriate antibiotics (100 μg/mL carbenicillin or 25 μg/mL kanamycin) and inducer (100 μM of 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was spread and dried on plates for blue/white-screening when appropriate.

For integration, NifS was cloned into pDSM-5a, NifU was cloned into pDSM-13, and SYN1 and SYN2 were cloned into an ampicillin resistant level-0 vector with BpiI (no. R0535L; New England Biolabs). High molecular weight DNA was isolated using the GeneOptimizer tool (ThermoFisher). All Sanger sequencing reactions were performed by Quintara Biosciences.

Plasmid isolations were performed with Qiagen Qiaprep kits. Genomic DNA was isolated using the Promega Wizard Genomic DNA Preparation Kit. Gel electrophoresis was carried out using 1% agarose Gels according to the lithium acetate method (35, 36). Chemically competent E. coli DH5α (New England Biolabs) was used as a cloning strain and grown at 37 °C in lysogeny broth media with appropriate antibiotics (100 μg/mL carbenicillin or 25 μg/mL kanamycin) and inducer (100 μM of 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was spread and dried on plates for blue/white-screening when appropriate.

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NiFBS were cloned into pDSM-ef. Six combinations of parental strains were created with varying promoters and terminators to vary expression levels as shown (Fig. 2A and SI Appendix, Fig. S1 A and B and Tables S1 and S2). To construct the library of NiFb variants, promoters Ptef1 and PPRX1 from S. cerevisiae were used for the high- and low-expression levels, respectively, and both used terminator TKIURA3 from Kluyveromyces lactis. A kanamycin-resistance gene was cloned into pDSM-bc with the strong promoter Pagtef1 and the TagTEFI terminator, both from Ashbya gossypii. These transcription units can recombine and integrate into yeast chromosome XV between NRT1 and GVP1 (starting at 459,247 bp end and at 458,821 bp). Integrations were performed using PCR-amplified transcription units (QS no. M04925; New England Biolabs) from the pDSM vectors, as well as PCR-amplified bridging DNA derived from the yeast genome. Parts were introduced to yeast by homologous recombination using the lactate acetyltransformation method (35, 36). Integrations were verified by whole-genome DNA extraction (Wizard no. A1125; Promega), PCR, and gel electrophoresis (1% agarose E-gel).

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**In Vitro FeMo-co Synthesis and Insertion Assays in Defined System Using Purified Proteins.** Assays were performed as described by Curatti et al. (3), with slight modifications. Unless specified, NiFb-dependent FeMo-co synthesis assays were performed in 100-μL reactions containing 17.5 μM NaMoO4, 175 μM R-homocitrato, 125 μM FeSO4, 125 μM NaS, 125 μM SAM, 1.23 mM ATP, 18 mM phosphocreatine disodium salt, 2.2 mM MgCl2, 3 mM DTH, 40 μM creatine phosphokinase, 5 μM NiFe, 3.0 μM NiFb, 1.5 μM apo-NiFEN, 3.0 μM NiFb, 0.6 μM apo-NiFDK, and 1 mg/mL bovine serum albumin in 22 mM Tris-HCl buffer (pH 7.5). FeMo-co synthesis and insertion into apo-NiFDK were performed under N2 atmosphere. In the absence of NiFb and SAM, and with NiFb isolated from yeast replacing the A. vinelandii NiFb in the above reaction. Following in vitro synthesis of FeMo-co, activation of apo-NiFDK was analyzed by addition of 500 μL of 2.0 mM NiFe and ATP-regenerating mixture (1.23 mM ATP, 18 mM phosphocreatine disodium salt, 2.2 mM MgCl2, 3 mM DTH, 40 μM creatine phosphokinase, final concentrations in 22 mM Tris-HCl [pH 7.5]). The assay (total volume in 9-mL vials) was prepared as above for 30 °C 15 min following standard procedures (37). Control reactions for acetylene reduction were carried out with purifications of A. vinelandii holo-NiFDK or apo-NiFDK activated using precursor-deficient apo-NiFEN supplemented with purified NiFb-co (25 μM Fe final concentration) (8). The purification of other proteins used in the assay has been previously described (14).

**EPR Analysis of NiFb.** NiFb preparations in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10% glycerol, 2 mM DTH, and 5 mM β-ME were used for EPR analysis. X-band (9.64 GHz) EPR spectra were recorded on a Bruker E500A spectrometer equipped with an Oxford ESR 910 cryostat for low-temperature measurements. The microwave frequency was calibrated with a frequency counter and the magnetic field with an NMR gauss meter. The temperature of the X-band cryostat was calibrated with a carbon-glass resistor temperature controller (LakeShore 341). For all EPR spectra a modulation frequency and amplitude of 100 kHz and 1 mT were used. The EPR spectra of Fig. 3E and SI Appendix, Fig. S14 were recorded at 12 K. EPR spectral simulations were performed using the simulation software Spin Count (38); 1 mM Cu(II)ethylenediaminetetraacetic acid solution is used as spin standard for spin quantification. Two EPR samples independently prepared from 2 different purifications, NiFb826 and NiFb82, were measured. Both purifications yielded very similar EPR signals. One set of data is presented in Fig. 3E, and both sets are presented in SI Appendix, Fig. S12.

**Data and Materials Availability.** All data are available in the main text, SI Appendix, or Dataset S1.

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