

# Heterologous transporters from anaerobic fungi bolster fluoride tolerance in *Saccharomyces cerevisiae*



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## ABSTRACT

Membrane-embedded transporters are crucial for the stability and performance of microbial production strains. Apart from engineering known transporters derived from model systems, it is equally important to identify transporters from nonconventional organisms that confer advantageous traits for biotechnological applications. Here, we transferred genes encoding fluoride exporter (FEX) proteins from three strains of early-branching anaerobic fungi (*Neocallimastigomycota*) to *Saccharomyces cerevisiae*. The heterologous transporters are localized to the plasma membrane and complement a fluoride-sensitive yeast strain that is lacking endogenous fluoride transporters up to 10.24 mM fluoride. Furthermore, we show that fusing an amino-terminal leader sequence to FEX proteins in yeast elevates protein yields, yet inadvertently causes a loss of transporter function. Adaptive laboratory evolution of FEX proteins restores fluoride tolerance of these strains, in one case exceeding the solute tolerance observed in wild type *S. cerevisiae*; however, the underlying molecular mechanisms and cause for the increased tolerance in the evolved strains remain elusive. Our results suggest that microbial cultures can achieve solvent tolerance through different adaptive trajectories, and the study is a promising step towards the identification, production, and biotechnological application of membrane proteins from nonconventional fungi.

## 1. Introduction

The performance of engineered microbial production strains can be dictated by membrane-embedded transporter proteins (Boyarskiy and Tullman-Ercek, 2015; Kell et al., 2015). For instance, product inhibition and toxicity is often mitigated via overexpression of drug exporters in biofuel-producing microbes (Dunlop et al., 2011; Foo et al., 2014; Teixeira et al., 2012). Directed evolution and rational protein engineering of known transporters offer a path to improve membrane protein function and ultimately strain performance (Farwick et al., 2014; Fisher et al., 2014; Foo and Leong, 2013; Mingardon et al., 2015). However, to increase the known transporter repertoire, it is valuable to mine transporters with potentially novel and improved functions from non-model organisms. Such approaches have identified transporters from various fungi and plants that were heterologously expressed in *Saccharomyces cerevisiae* to engineer strains capable of utilizing sucrose (Marques et al., 2018), xylose (Runquist et al., 2010; Saloheimo et al., 2007), and cellobiose (Sadie et al., 2011), and these heterologous transporters have been further improved by classic directed evolution strategies (Wang et al.,

2016; Young et al., 2012).

Anaerobic gut fungi (*Neocallimastigomycota*) are an underexplored source of biotechnologically interesting enzymes and membrane transporters with direct application to lignocellulosic breakdown and hydrolysate fermentation. These early diverging fungi inhabit the digestive systems of large herbivores, where they secrete powerful cellulolytic enzymes that aid in the digestion of recalcitrant, lignin-rich plant fibers (Gordon and Phillips, 1998; Solomon et al., 2016; Theodorou et al., 1996). Because of their unusual habitat and diverse enzymatic secretome, it is likely that anaerobic fungi harbor specialized membrane transporters that allow them to utilize biohydrolysates and thrive in the rumen microbiome. This is corroborated by the recent genomic and transcriptomic sequencing of four different gut fungal strains, revealing a large repertoire of putative membrane-embedded receptors and transporters that are likely involved in their unusual lifestyle (Haitjema et al., 2017; Henske et al., 2017; Seppälä et al., 2016). However, owing to their complex native environment and handling requirements, anaerobic gut fungi are challenging to maintain in the laboratory (Haitjema et al., 2014), and their membrane proteome has not been functionally explored

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or tested. Apart from the expression of genes encoding ADP/ATP carriers from *Neocallimastix* sp. in *Escherichia coli* and *S. cerevisiae* more than a decade ago, no gut fungal membrane proteins have been heterologously produced or characterized (Haferkamp et al., 2002; van der Giezen et al., 2002; Voncken et al., 2002). Therefore, a wealth of potentially industrially relevant gut fungal membrane proteins remain untested, resulting in a knowledge gap regarding their relative activity and potential for use in model systems.

Here, as proof-of-principle, we show that bioinformatically identified fluoride transporters from three strains of anaerobic fungi can be transferred to a model yeast system and restore fluoride tolerance in a fluoride-sensitive knockout yeast. Fluoride is a ubiquitous xenobiotic that adversely affects several cellular processes (Barbier et al., 2010; Zuo et al., 2018). Consequently, most organisms require fluoride ion exporters to maintain intracellular fluoride concentrations below toxic levels (Baker et al., 2012; Barbier et al., 2010). In this study, we identify and produce functional fluoride exporter (FEX) proteins from *Neocallimastix californiae*, *Piromyces finnis* and *Anaeromyces robustus* in *S. cerevisiae*. Upon codon optimization, the putative FEX genes are highly expressed, and the membrane proteins are targeted to the endoplasmic reticulum and trafficked to the plasma membrane. The addition of an amino-terminal leader peptide improves total protein titers but reduces tolerance to fluoride. Subsequent evolutionary engineering of strains harboring heterologous FEX proteins not only restores, but improves both protein yields and fluoride tolerance through apparently divergent evolutionary paths. In one case, cellular fluoride tolerance is increased to a level greater than that observed in wildtype yeast. These results are the first to demonstrate heterologous production of a plasma membrane-embedded ion transporter from anaerobic gut fungi, and demonstrate the utility of these transporters for strain engineering.

## 2. Materials and methods

### 2.1. Identification and cloning of fluoride exporter (FEX) genes

Genes encoding FEX proteins were identified in transcriptomic and genomic data collected from 3 strains of anaerobic gut fungi (Haitjema et al., 2017; Seppälä et al., 2016; Solomon et al., 2016). The topology of the proteins was predicted using TMHMM (Krogh et al., 2001). Alignments were made using PSI/TM-Coffee and the ExPASy Boxshade tool (Artimo et al., 2012; Chang et al., 2012). The genes were synthesized by Genewiz (South Plainfield, NJ, USA); the *P. finnis* and *A. robustus* FEX genes were codon optimized for expression in *E. coli* whereas the *N. californiae* FEX gene was synthesized both in a codon-optimized version and a non-codon optimized version. The genes were subsequently cloned into the yeast centromeric pYC2/CT vector, using restriction enzymes EagI and XhoI and T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). In the pYC vector, the cloned gene is downstream of a *GAL1* promoter and 3'-terminally fused to green fluorescent protein followed by a decahistidine tag. In a subset of pYC vectors, the gut fungal FEX gene is preceded by a 5' Preproleader sequence, which, including a 3' cloning scar, translates into the following peptide: MKVLIVLLAIFAALPLALAQPVISTTVGSAEGLDKREARPDV (Clements et al., 1991). The gene encoding Fex1p was amplified from the *S. cerevisiae* genome using primers 1 and 2 (Supplementary Table S3) and subcloned into pYC2/CT. DNA- and PCR-purification kits were from Zymo Research (Irvine, CA). Primers were from Eurofins MWG Operon, KY, USA. Plasmids were verified by Sanger sequencing (Genewiz, South Plainfield, NJ, USA).

### 2.2. Construction of FEX deletion mutants

The *fex1:GSHU* *fex2Δ* deletion strain was generated in the background of *S. cerevisiae* BJ5465 (MATa *ura3-52* *trp1* *leu2Δ1* *his3Δ200* *pep4:HIS3* *prb1Δ1.6R* *can1* *GAL*) (ATCC, 208289) using the *delitto perfetto* method, as described previously (Stuckey and Storici, 2013). The

*fex1:GSHU* mutation was constructed by integrating the GSHU gene cassette into the *FEX1* locus using primers 3 and 4. The *fex2* knockout was constructed by integrating the CORE-Kp53 gene cassette into the *FEX2* locus using primers 3 and 5 and subsequently removing the cassette using primers 6 and 7. Gene insertions and deletions were verified by PCR and Sanger sequencing (Genewiz, South Plainfield, NJ, USA).

### 2.3. Transformation and FEX expression in yeast

pYC2/CT plasmids were transformed into *S. cerevisiae* BJ5465 *fex:GSHU* *fex2Δ* using the lithium-acetate/PEG method (Gietz, 2014). Transformants were selected on SD-URA plates (20 g/L D-glucose, 6.7 g/L yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD, USA), 5 g/L casamino acids (Becton, Dickinson & Co., Sparks, MD, USA), 40 mg/L Tryptophan, 40 mg/L Adenine, 16.25 g/L sodium citrate dihydrate, 4.2 g/L citric acid monohydrate, 20 g/L agar (Becton, Dickinson & Co., Sparks, MD, USA)). Genes were expressed essentially as described in Drew et al., with the following modifications (Drew et al., 2008). Briefly, individual colonies were used to inoculate 3 mL overnight cultures in SD-URA medium (20 g/L D-glucose, 6.7 g/L yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD, USA), 5 g/L casamino acids (Becton, Dickinson & Co., Sparks, MD, USA), 40 mg/L Tryptophan, 40 mg/L Adenine, 16.25 g/L sodium citrate dihydrate, 4.2 g/L citric acid monohydrate) and grown at 30 °C, 225 rpm, for ~16 h. The overnight cultures were used to seed an expression culture in SR-URA medium (same composition as SD-URA, except with 20 g/L raffinose instead of glucose) to a starting OD<sub>600</sub> of 0.12. The culture was grown at 30 °C, 225 rpm for 6–7 h or until OD<sub>600</sub> ~0.6–1, when gene expression was induced with 2% w/v galactose for 22–24 h (30 °C, 225 rpm).

### 2.4. Confocal microscopy

0.1 ODU of induced yeast culture was centrifuged and resuspended in 1X PBS buffer. Samples were transferred to wells of Nunc Lab-Tek chambered slides coated with 0.1% (w/v) poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA). Microscopy was performed using an Olympus Fluoview 1000 Spectral confocal microscope with a 60x/NA 1.3 objective. GFP was visualized using a 488 nm laser and 530/20 nm bandpass filter. In order to more clearly display protein trafficking patterns, the laser intensity was adjusted for each strain to bring fluorescence intensities near saturating levels.

### 2.5. Fluorescence-activated cell sorting (FACS)

FEX gene expression was induced as described above. An equivalent of 0.2 ODU was pelleted at 3000 g for 30s and resuspended in 200 μL 1X PBS prior to FACS analysis. Scatter and fluorescence intensity were measured for approximately 60,000 singlet cells from each sample using a BD FACSARIA I flow cytometer. Fluorescence intensity from GFP was determined using a 488 nm laser and 530/30 nm bandpass filter.

### 2.6. In vivo fluoride tolerance assay and adaptive laboratory evolution

2 ODU of induced yeast culture was collected by centrifugation at 3000 × g, 2 min, and resuspended in 40 μL 1x PBS buffer. 3 μL of the cell suspension (0.15 ODU) was streaked on an SG-URA plate (20 g/L galactose, 6.7 g/L yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD, USA), 5 g/L casamino acids (Becton, Dickinson & Co., Sparks, MD, USA), 40 mg/L Tryptophan, 40 mg/L Adenine, 16.25 g/L sodium citrate dihydrate, 4.2 g/L citric acid monohydrate, 20 g/L agar (Becton, Dickinson & Co., Sparks, MD, USA)) supplemented with 50 μM NaF. Plates were incubated at 30 °C for approximately 48 h. For liquid assay, cultures were induced to trigger FEX expression as described above. The induced cultures were used to seed 3 mL cultures in SRG-URA medium (20 g/L raffinose, 20 g/L galactose, 6.7 g/L yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD, USA), 5 g/L casamino acids (Becton, Dickinson

& Co., Sparks, MD, USA), 40 mg/L Tryptophan, 40 mg/L Adenine, 16.25 g/L sodium citrate dihydrate, 4.2 g/L citric acid monohydrate), supplemented with increasing concentrations of NaF (0–81.96 mMNaF), at a starting  $OD_{600} = 1$ . The cultures were incubated at 30 °C, 225 rpm, for 22–24 h. Growth was determined by measuring the optical density at 600 nm. For adaptive laboratory evolution, induced cultures were used to seed 3 mL of SRG-URA medium supplemented with 0.020 mM and 0.040 mM fluoride, at a starting  $OD_{600} = 1$ . The cultures were incubated at 30 °C, 225 rpm. After 16–48 h,  $OD_{600}$  was measured and the cultures were used to seed 3 mL SRG-URA medium supplemented with the highest tolerated concentration of fluoride at which growth could be detected, as well 3 mL SRG-URA medium supplemented with twice the highest tolerated concentration of fluoride, in both cases at a starting  $OD_{600} = 1$ . This serial seeding into increasingly higher concentrations of fluoride was repeated over a period of ~21 days.

### 2.7. Plasmid extraction and curing of evolved strains

Plasmids were extracted from the evolved yeast strains using a Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research (Irvine, CA)). Isolated plasmids were propagated and extracted from *E. coli* DH5 $\alpha$  and verified by Sanger sequencing (Genewiz, South Plainfield, NJ, USA). In order to cure evolved yeast strains of plasmid, cells were plated onto SD plates (20 g/L D-glucose, 6.7 g/L yeast nitrogen base (Becton, Dickinson & Co., Sparks, MD, USA), 5 g/L casamino acids (Becton, Dickinson & Co., Sparks, MD, USA), 40 mg/L Tryptophan, 40 mg/L Adenine, 40 mg/L uracil, 16.25 g/L sodium citrate dihydrate, 4.2 g/L citric acid monohydrate, 20 g/L agar (Becton, Dickinson & Co., Sparks, MD, USA)) containing the auxotrophic marker (uracil) encoded in the pYC plasmid backbone. Colonies were replica-plated onto SD and SD-URA plates to identify those unable to grow in the absence of uracil due to loss of plasmid. The Ura<sup>-</sup> phenotype was verified by confirming growth or lack of growth after overnight incubation in 3 mL SD and SD-URA cultures, respectively.

## 3. Results and discussion

### 3.1. Identification and heterologous expression of anaerobic gut fungal FEX homologs

Genomic and transcriptomic data suggest that each of the early diverging fungal strains *N. californiae*, *P. finnis*, and *A. robustus* express one gene encoding a FEX protein homolog that is predicted to extrude fluoride ions from cells (Fig. 1A) (Haitjema et al., 2017; Seppälä et al., 2016). The gut fungal FEX proteins share ~65% of their amino acid composition and are only ~30% identical to the *S. cerevisiae* homologs Fex1p and Fex2p, which are virtually identical to each other (Supplementary Figure S1). Similar to other eukaryotic FEX proteins, the gut

fungal proteins are predicted to have 9 transmembrane helices and two Fluc domains (PF02537) (Supplementary Figure S2) (Li et al., 2013; Rapp et al., 2006; Smith et al., 2015; Stockbridge et al., 2013).

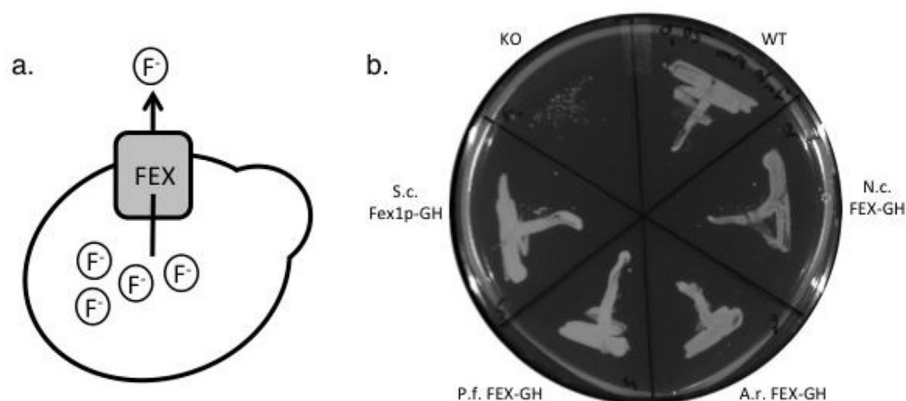
Three gut fungal FEX genes identified from *N. californiae*, *P. finnis* and *A. robustus* transcriptomes were synthesized and cloned into the centromeric yeast vector pYC2/CT with a carboxy (C)-terminal GFP-His<sub>10</sub> (GH) tag to determine whether heterologous FEX transporters could be produced in yeast. Compared to most model organisms, gut fungal genomes are extremely AT-rich (Haitjema et al., 2017; Nicholson et al., 2005; Youssef et al., 2013): the GC-content of the FEX genes is 25–28%, necessitating codon optimization prior to expression in *S. cerevisiae* (Supplementary Table S1). The genes were initially optimized for expression in *E. coli*, but upon induction the growth of the bacterial cultures was severely impaired implying that the FEX proteins were toxic to *E. coli* possibly due to improper targeting, folding, or post-translational modification of the eukaryotic membrane proteins (not shown) (Wagner et al., 2007).

SDS-PAGE analysis of yeast membranes suggests that the gut fungal FEX proteins are produced in the Pep4 protease-deficient yeast strain BJ5465 with minimal degradation or aggregation (Supplementary Figure S3). The expected sizes of the proteins, including the C-terminal GFP-His<sub>10</sub> tag, are: 64.6 kDa (*N. californiae* FEX-GH), 63.5 kDa (*P. finnis* FEX-GH) and 63.9 kDa (*A. robustus* FEX-GH), but as often noted for hydrophobic,  $\alpha$ -helical membrane proteins, the FEX proteins migrate somewhat faster than expected compared to conventional molecular weight standards (Rath et al., 2009).

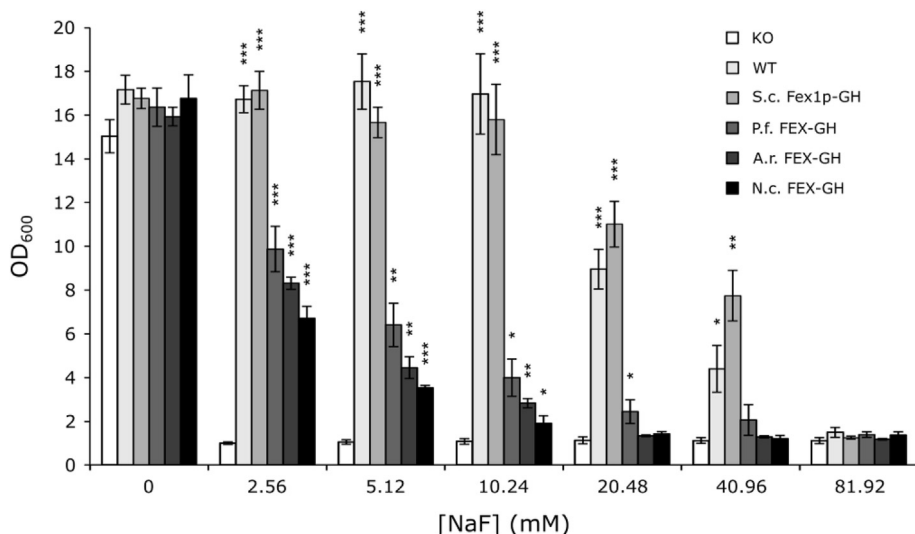
### 3.2. Gut fungal FEX homologs complement a fluoride sensitive yeast strain

*S. cerevisiae* has two endogenous genes encoding FEX proteins (YOR39OW, Fex1p; and YPL279C, Fex2p); the proteins are virtually identical and both genes must be knocked out for yeast to exhibit fluoride sensitivity (Li et al., 2013). To assess whether the gut fungal proteins are functional in *S. cerevisiae*, we constructed a fex knock out (KO) strain (BJ5465 *fex1:GSHU* $\Delta$ *fex2*), which fails to grow when exposed to  $\mu$ M concentrations of fluoride (Fig. 1B). The KO strain was subsequently transformed with plasmids encoding gut fungal FEX genes and plated on induction medium containing 50  $\mu$ M NaF. All three gut fungal proteins restore fluoride tolerance in the KO strain (Fig. 1B).

To quantify the degree of fluoride tolerance, we tested the viability of each FEX-producing yeast strain (both endogenous and heterologous) in liquid cultures with increasing concentrations of fluoride. Similar to previous reports, the KO strain exhibits high sensitivity to fluoride resulting in insignificant growth in the lowest NaF concentration tested (Li et al., 2013). In contrast, the wild type strain and the KO strain producing plasmid-borne *S. cerevisiae* Fex1p-GH demonstrate unimpaired growth in the presence of up to 10.24 mM NaF and attenuated growth in up to 40.96 mM NaF (Supplementary Table S2 and Fig. 2). The activity



**Fig. 1.** Fex proteins extrude fluoride from cells. (a) Cartoon depiction of a cell producing a fluoride transporter (FEX). (b) Anaerobic fungal FEX transporters restore fluoride tolerance in a yeast knockout (KO) strain. 0.15 ODU of induced yeast cultures were harvested and plated on SG-URA supplemented with 2% w/v galactose and 50  $\mu$ M NaF. Streaked plates were incubated at 30 °C for approximately 48 h. KO = BJ5465 *fex1:GSHU*  $\Delta$ *fex2* carrying the empty pYC vector. WT = BJ5465 carrying the empty pYC vector. N. c. FEX-GH = KO with pYC-*N. californiae* FEX-GH. A. r. FEX-GH = KO with pYC-*A. robustus* FEX-GH. P. f. FEX-GH = KO with pYC-*P. finnis* FEX-GH. S. c. Fex1p-GH = KO with pYC-*S. cerevisiae* Fex1p-GH.

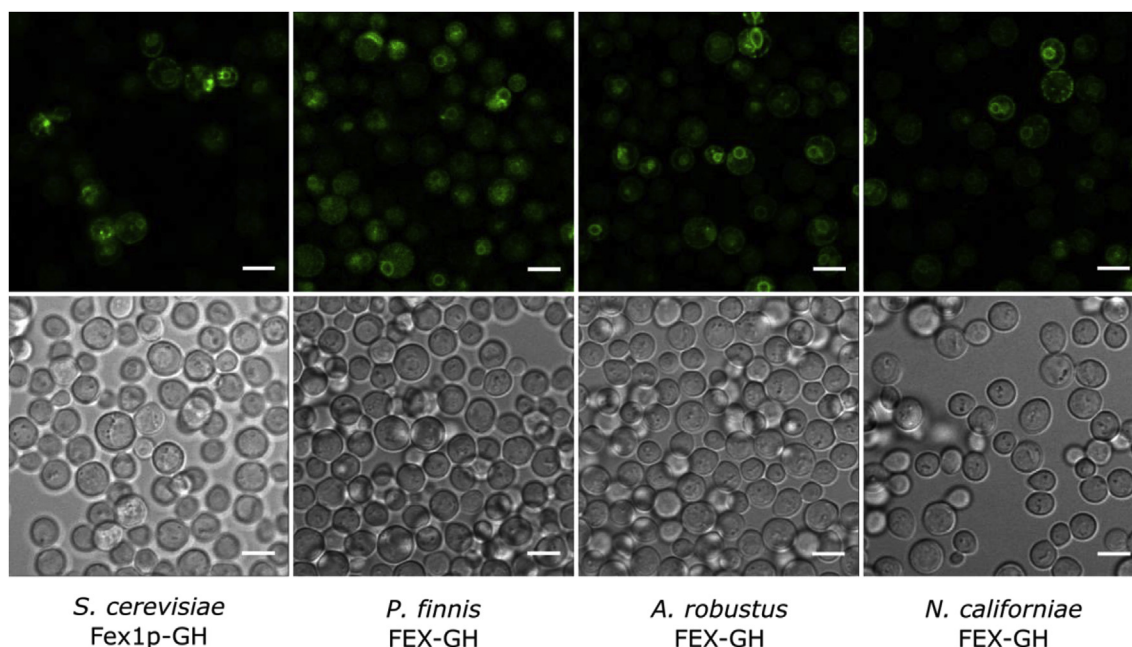


**Fig. 2. KO-strains harboring anaerobic fungal FEX proteins show attenuated growth in up to 10.24 mM NaF.** OD<sub>600</sub> values of indicated yeast strains were measured after ~22 h growth in the presence of inducer (2% w/v galactose) and increasing concentrations of NaF. Wildtype (WT) and a KO strain expressing *S. cerevisiae* Fex1p-GH exhibit growth in up to 40.96 mM NaF. In contrast, KO strains expressing gut fungal homologs show reduced fluoride tolerance exhibiting growth in up to 10.24 mM NaF. Indicated is the standard error of the mean of three biological replicates. Statistically significant differences in growth between FEX-producing strains and the KO control strain were determined using a one-tailed Student's t-test: \*\*\* =  $p$ -value < 0.001, \*\* =  $p$ -value < 0.01, \* =  $p$ -value < 0.05. KO = BJ5465 *fex1:GSHU Δfex2* carrying the empty pYC vector. WT = BJ5465 carrying the empty pYC vector. *S. c.* Fex1p-GH = KO with pYC *S. cerevisiae* Fex1p-GH. *P. f.* FEX-GH = KO with pYC *P. finnis* FEX-GH. *A. r.* FEX-GH = KO with pYC *A. robustus* FEX-GH. *N. c.* FEX-GH = KO with pYC *N. californiae* FEX-GH.

observed for *S. cerevisiae* Fex1p-GH contradicts a previous report in which a C-terminal GFP tag significantly reduced the activity of Fex1p (Smith et al., 2015). The activity observed in this work may reflect differences in gene copy number and promoter strength as Smith et al. expressed *FEX1* from the native locus under the control of the native promoter, whereas our system utilizes the strong, inducible  $P_{GAL1}$  promoter and a centromeric plasmid with an expected copy number of 1–2 (Clarke and Carbon, 1980). As a result of increased copy number and transcription, functional protein yield may be increased in our system. All strains producing gut fungal FEX-GH proteins exhibit attenuated growth in the presence of 5.12–10.24 mM NaF demonstrating restoration of fluoride tolerance in the KO strain, albeit to levels below that of the WT strain.

### 3.3. Gut fungal FEX homologs are trafficked to the plasma membrane

Yeast strains were analyzed using confocal microscopy and flow cytometry to assess the impacts of membrane protein trafficking and yield on the fluoride tolerance of the strains. In *S. cerevisiae*, targeting to cytoplasmic proteasomes (via the ERAD pathway) is often a consequence of protein misfolding and stacking in the endoplasmic reticulum (ER) (Delic et al., 2013). Confocal microscopy reveals that all gut fungal FEX-GH proteins are targeted to the perinuclear ER and the cell periphery in a pattern similar to the native *S. cerevisiae* Fex1p-GH (Fig. 3). Additionally, FEX-GH proteins may partially reside in the cortical ER as supported by the observation of punctuate fluorescent structures at the cell periphery. Although protein retention in the cortical ER is plausible, the cellular activity demonstrated in growth assays is indicative of properly



**Fig. 3. Confocal micrographs show localization of GFP-tagged FEX proteins produced in a *S. cerevisiae* knockout strain.** Yeast knockout strains producing the indicated FEX homologs exhibit localization of fluorescent protein to the endoplasmic reticulum and plasma membrane signifying proper membrane protein folding and targeting. GFP (top) and transmission (bottom) micrographs were obtained in the absence of fluoride after ~22–24 h induction of *FEX* gene expression in raffinose- and galactose-based medium. Microscopy was performed using an Olympus Fluoview 1000 Spectral confocal microscope equipped with a 60x/NA 1.3 objective. Laser intensities were adjusted for each strain to bring fluorescence intensities near saturating levels. All scale bars represent 5 μm.

trafficked, active FEX proteins at the cell membrane.

Analysis of recombinant *S. cerevisiae* strains using flow cytometry demonstrates appreciable yields of each gut fungal homolog (Fig. 4). All three strains exhibit mean fluorescence intensities (MFI) ~4 fold over background autofluorescence, approximately half of the value observed upon expression of *S. cerevisiae* FEX1p-GH from the same plasmid backbone. The decreased MFI is indicative of a reduction in total protein yield compared to *S. cerevisiae* Fex1p-GH. Given the similar trafficking patterns, the reduced protein yields may underlie the lower fluoride tolerance associated with strains producing gut fungal FEX proteins.

### 3.4. Addition of a synthetic leader peptide increases FEX protein levels but decreases cellular fluoride tolerance

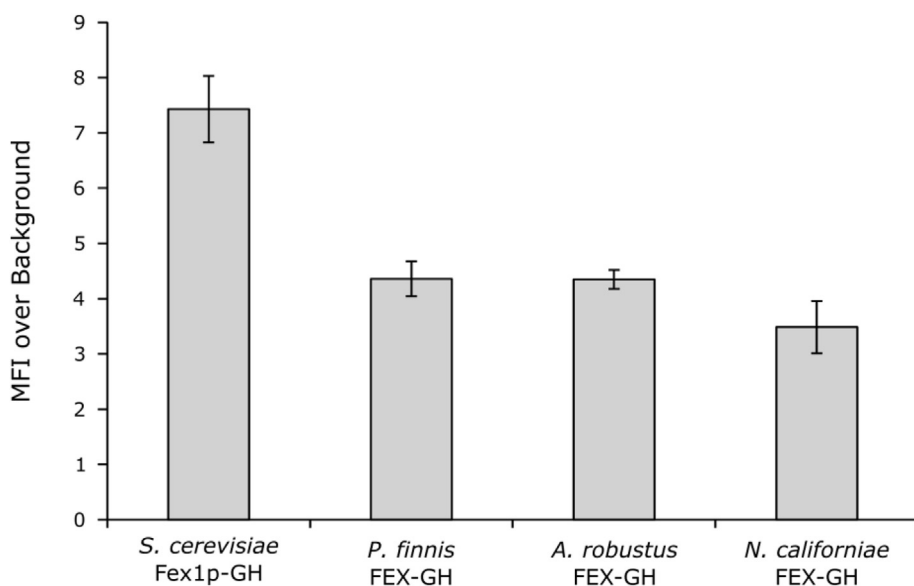
As both the total protein yields and fluoride tolerance were lower for strains producing gut fungal FEX proteins compared to the wild-type yeast Fex1p, we hypothesized that we could improve FEX-GH trafficking, functional yield, and concomitant fluoride tolerance through the addition of an amino-terminal leader peptide. The synthetic Prepro leader peptide, consisting of a consensus eukaryotic signal peptide followed by a linker sequence and a KEX2 protease site, has been successfully used in yeast to promote targeting and trafficking of recombinant proteins to the plasma membrane (Arnold et al., 1998; Clements et al., 1991; O'Malley et al., 2009; von Heijne, 1985). Accordingly, the gene encoding this peptide was cloned upstream of the FEX-GH coding sequences and the resulting constructs were transformed into the KO strain. Analysis of strains producing Prepro-FEX-GH using confocal microscopy suggests trafficking patterns similar to those producing Prepro-less proteins (Supplementary Figure S4). In all strains, fluorescence is localized to the perinuclear ER and cell periphery; however, the relative fluorescence intensities, particularly at the periphery, appear to be greater in these strains. Flow cytometry corroborates the observation of increased cellular fluorescence intensity; yeast strains producing amino-terminally modified FEX proteins exhibit ~11-fold MFI over background representing ~3-fold increases compared to strains producing unmodified FEX proteins (Fig. 5a).

While addition of the Prepro peptide improves total protein yields, cellular fluoride tolerance is considerably diminished, and we observe attenuated growth of all strains cultured in media containing up to 0.32 mM NaF (Supplementary Table S2 and Fig. 5b). The Prepro domain is expected to integrate into the ER membrane and undergo subsequent peptidase cleavage (Arnold et al., 1998; Clements et al., 1991; O'Malley

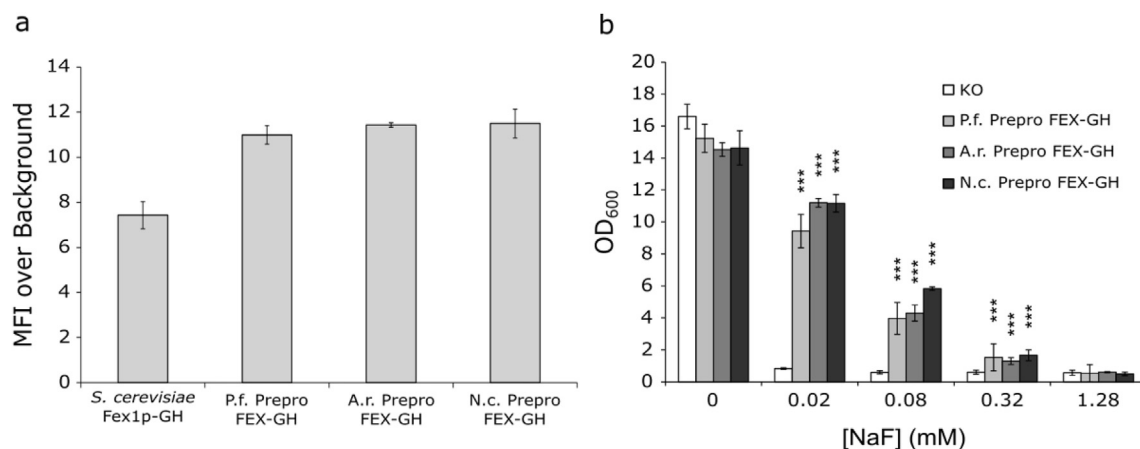
et al., 2009; von Heijne, 1985), but SDS-PAGE analysis suggests that the leader peptide preceding the FEX proteins is not processed (the unprocessed Prepro peptide adds ~4 kDa to the molecular weight of the protein) (Supplementary Figure S3). Therefore, we speculate that the Prepro peptide may interfere with insertion, folding, or function of the downstream protein. Alternatively, the uncleaved signal peptide may be indicative of increased protein retention in the ER, which may reduce functional protein yield in at least two ways. First, the unfolded protein response (UPR) may be induced or exacerbated leading to decreases in functional protein as reported for the overexpression of a heterologous P2 H<sup>+</sup>/adenosine transporter in *S. cerevisiae* (Griffith et al., 2003). Second, increased retention in the ER may reduce the amount of protein trafficked to the plasma membrane.

### 3.5. Evolutionary engineering significantly improves fluoride tolerance of prepro-FEX-GH-producing strains

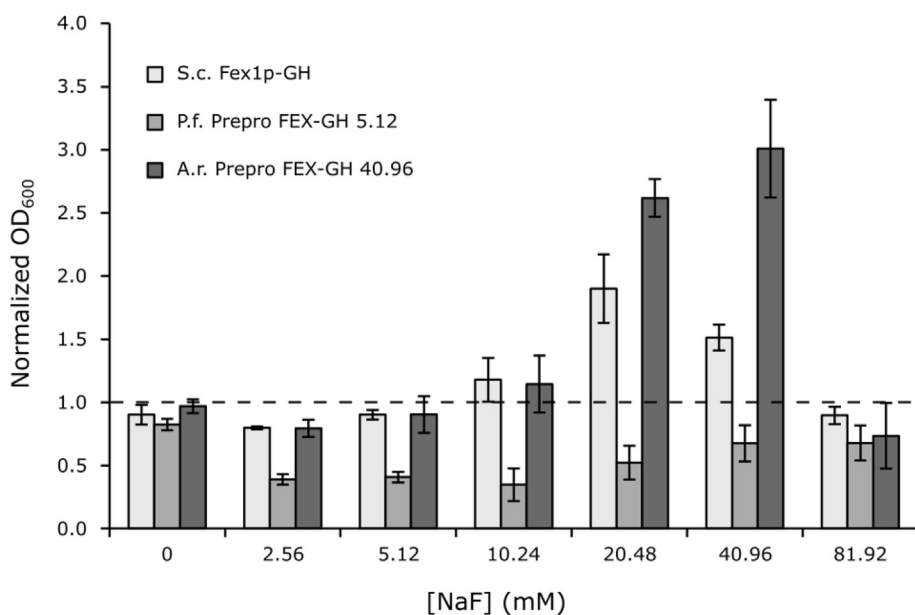
In order to improve cellular fluoride tolerance, we performed an evolutionary engineering experiment wherein strains producing gut fungal Prepro-FEX-GH were propagated in serial batch cultures containing increasing concentrations of fluoride. To challenge the strains to a greater extent and potentially identify mutations resulting in greater phenotypic improvements, the experiment was carried out using strains producing Prepro-FEX-GH, which exhibit high total protein yields but low fluoride tolerance. Starting with a culture supplemented with low ( $\ll$  1 mM) concentrations of fluoride, we serially seeded cultures with increasingly higher concentrations of fluoride and quickly isolated two strains that are able to grow in significantly higher concentrations of fluoride than their parent strains: *P. finnis* Prepro-FEX-GH 5.12 exhibits attenuated growth in up to 5.12 mM NaF, a 16-fold improvement, whereas *A. robustus* Prepro-FEX-GH 40.96 tolerates 40.96 mM NaF, which is greater than the concentration toxic to wild type yeast and represents a 128-fold increase in fluoride tolerance (Supplementary Table S2 and Supplementary Figure S5). Remarkably, in 40.96 mM NaF, the evolved strain producing *A. robustus* Prepro-FEX-GH grows to an OD<sub>600</sub> value ~3-fold greater than WT yeast (Fig. 6). For comparison, a KO strain producing *S. cerevisiae* Fex1p-GH from the same vector backbone, thus providing a closer comparison with respect to promoter strength and gene copy number, grows to a final OD<sub>600</sub> value ~1.5-fold greater than WT yeast. These results demonstrate the potential value of novel transporters mined from anaerobic fungi and the capacity to evolve improved cellular function through relatively simple methods such as



**Fig. 4. Knockout strains producing gut fungal FEX-GH proteins exhibit decreased total protein yield compared to a strain producing *S. cerevisiae* Fex1p-GH.** Analysis of strains using fluorescence-activated cell sorting (FACS) provides a proxy measurement of total protein yield. Compared to a knockout strain expressing the native *S. cerevisiae* Fex1p-GH from a low copy vector, strains expressing gut fungal homologs from the same vector backbone display lower cellular mean fluorescence intensities (MFI). Strains were cultured and gene expression was induced in the absence of fluoride. Indicated is the standard deviation from the mean of three biological replicates. For each replicate, fluorescence data was collected for ~40,000 single cells.



**Fig. 5. Addition of Prepro leader peptide to FEX-GH proteins leads to improved total protein yields and reduced cellular fluoride tolerance.** (a) Knockout strains producing Prepro-FEX-GH homologs in the absence of fluoride exhibit cellular MFI values significantly greater than the strain producing *S. cerevisiae* Fex1p-GH. The increase in cellular MFI relative to *S. cerevisiae* Fex1p-GH is indicative of improved protein yields, suggesting a beneficial effect arises due to addition of Prepro. (b) The strains producing Prepro-FEX-GH homologs exhibit low tolerances to NaF suggesting that the Prepro peptide is deleterious to protein function. Indicated is the standard deviation from the mean of three biological replicates. For each replicate in panel a, fluorescence data was collected for ~40,000 single cells. In panel b, OD<sub>600</sub> values of KO strains expressing *Prepro-FEX-GH* were measured after ~22–24 h growth in the presence of inducer (2% w/v galactose) and increasing concentrations of NaF. Statistically significant differences in growth between FEX-producing strains and the KO control strain were determined using a one-tailed Student's t-test: \*\*\* =  $p$ -value < 0.001, \*\* =  $0.001 < p$ -value < 0.01, \* =  $0.01 < p$ -value < 0.05. KO = BJ5465 *fex1:GSHU Δfex2* carrying the empty pYC vector. *P. f.* FEX-GH = KO with pYC *P. finnis* FEX-GH. *A. r.* FEX-GH = KO with pYC *A. robustus* FEX-GH. *N. c.* FEX-GH = KO with pYC *N. californiae* FEX-GH.

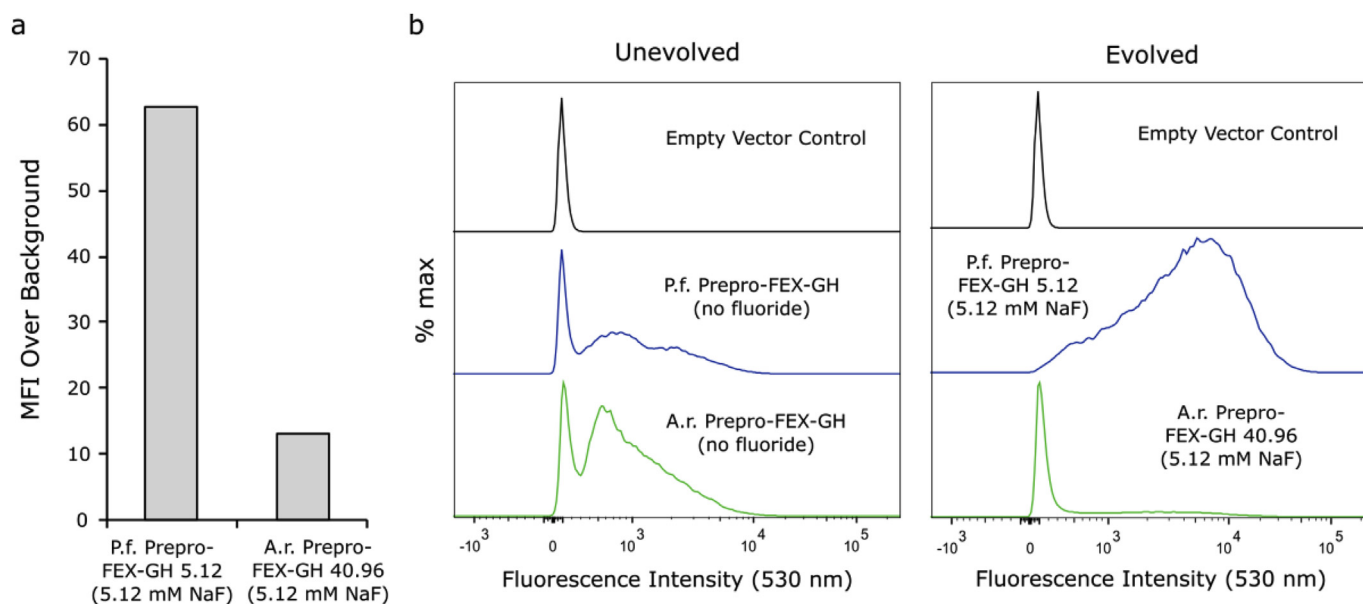


**Fig. 6. Evolved Prepro-FEX-producing strains exhibit distinct changes in fluoride tolerance.** Following adaptive evolution, the OD<sub>600</sub> values of evolved strains were measured and normalized to the OD<sub>600</sub> of a wildtype BJ5465 empty vector control grown at each NaF concentration. A KO strain producing *S. cerevisiae* Fex1p-GH provides a reference controlling for promoter strength and a closer comparison with respect to transporter gene copy number. This strain exhibits comparable or greater growth compared to WT yeast at all but the highest NaF concentrations tested. Similar to *S. cerevisiae* Fex1p-GH, *A. robustus* Prepro-FEX-GH 40.96 grows to cell densities comparable to, or greater than, WT at all but the highest NaF concentrations. In contrast, *P. finnis* Prepro-FEX-GH 5.12 grows to a lower concentration than WT yeast in all cases. OD<sub>600</sub> values of evolved KO strains were measured after ~24 h growth in the presence of inducer (2% w/v galactose) and increasing concentrations of NaF. Indicated is the standard error of the mean of three biological replicates. KO = BJ5465 *fex1:GSHU Δfex2* carrying the empty pYC vector. *S. c.* Fex1p-GH = KO with pYC *S. cerevisiae* Fex1p-GH. *P. f.* FEX-GH = KO with pYC *P. finnis* FEX-GH. *A. r.* FEX-GH = KO with pYC *A. robustus* FEX-GH.

adaptive laboratory evolution.

There are several paths that may lead to increased cellular fitness in the presence of fluoride: improved FEX activity, greater yields of functional FEX proteins, altered membrane composition reducing HF influx, etc. Analysis of *P. finnis* Prepro-FEX-GH 5.12 in the presence of 5.12 mM NaF using flow cytometry reveals a 5.7-fold increase in cellular MFI (Fig. 7a) as well as a significant improvement in phenotypic homogeneity (Fig. 7b). This phenotype is similar in the presence of 10.24 mM NaF (Supplementary Figure S6). These changes suggest that improved protein yield contributes to the apparent improvement in fluoride tolerance compared to the unevolved strain. SDS-PAGE of membranes containing *P. finnis* Prepro-FEX-GH 5.12 corroborates the elevated protein yields observed using flow cytometry (Supplementary Figures S3). As noted previously, the addition of the Prepro peptide may have exacerbated the

cellular UPR possibly resulting in increased FEX proteolysis via the ERAD pathway. It is possible that the cells reduced UPR (e.g., through increased chaperone protein production), resulting in improved protein yield. Additional experiments quantifying the degree of UPR induced in each strain would provide insight on this hypothesis. By sequencing plasmids extracted from the evolved strains, we ruled out beneficial mutations in the *FEX* coding sequences or flanking regulatory regions as the cause for increased fluoride tolerance. Notably, transformation of plasmid extracted from *P. finnis* Prepro-FEX-GH 5.12 into the original knock out strain resulted in fluoride tolerance comparable to the unevolved *P. finnis* Prepro-FEX-GH strain, suggesting that the plasmid in the evolved strain is not the sole cause of the improved phenotype (data not shown). Furthermore, curing the evolved strain of plasmid and re-transforming this cured, evolved strain with the original *P. finnis* Prepro-FEX-GH



**Fig. 7. Evolved Prepro-FEX-producing strains exhibit divergent fluorescence phenotypes.** (a) *P. finnis* Prepro-FEX-GH 5.12 exhibits significantly improved MFI relative to its parent strain indicative of increased total protein yield. In contrast, the MFI of *A. robustus* Prepro-FEX-GH 40.96 is similar to that of the unevolved strain. (b) Histograms reveal phenotypic heterogeneity at the population level. While *P. finnis* Prepro-FEX-GH 5.12 exhibits complete homogeneity in the presence of NaF, *A. robustus* Prepro-FEX-GH 40.96 displays increased heterogeneity. Fluorescence data represents the mean fluorescence intensity corresponding to ~40,000 single cells. KO = BJ5465 *fex1:GSHU Δfex2* carrying the empty pYC vector. P. f. FEX-GH = KO with pYC *P. finnis* FEX-GH. A. r. FEX-GH = KO with pYC *A. robustus* FEX-GH.

plasmid did not result in any elevated fluoride tolerance. We conclude that while the fluoride tolerance of the evolved strain may be a result of genetic mutations outside the regions we screened, they may also be a result of reversible adaptation. For example, Schütz *et al.* used directed evolution to increase yields of functional G protein-coupled receptor in yeast; however, the yields decreased after repeated culturing or transformation of the receptor into fresh yeast cells (Schütz *et al.*, 2016). In contrast, flow cytometric analysis of *A. robustus* Prepro-FEX-GH 40.96 in the presence of 5.12 mM NaF suggests an alternative path towards improved cellular fitness. These cells exhibit a 1.1-fold increase in MFI and greater phenotypic heterogeneity despite 2 orders of magnitude improvement in fluoride tolerance (Fig. 7). A similar phenotype is observed in the presence of 10.24 mM NaF (Supplementary Figure S6). Moreover, in the absence of fluoride, *A. robustus* Prepro-FEX-GH 40.96 exhibits basal fluorescence intensities. The low fluorescence intensity exhibited by *A. robustus* Prepro-FEX-GH 40.96 suggests decreased production of protein or post-translational proteolysis or misfolding of the GFP tag. Interestingly, in-gel fluorescence suggests that there is a low amount of full-length GFP-tagged protein and no free GFP could be detected (Supplementary Figure S3). Thus, the mechanism by which this strain has acquired its robust tolerance towards fluoride remains unknown.

#### 4. Conclusions

In this work, we produce functional, recombinant gut fungal membrane proteins in *S. cerevisiae* to enhance ion tolerance. To our knowledge, this is the first study to demonstrate the industrial utility of membrane proteins from the early-diverging *Neocallimastigomycota* fungi. Further, gut fungal protein yields and cellular ion tolerance exceeded wildtype levels through fusion of an amino-terminal leader peptide combined with adaptive laboratory evolution. Taken together, these results show that heterologous transporters from anaerobic fungi improve the phenotype of an industrially relevant model microorganism. Moreover, this effort represents a critical first step towards the identification, characterization and, ultimately, utilization of membrane proteins from a class of eukaryotic microorganisms that specialize in biomass

degradation and possess a wealth of biotechnologically promising yet completely uncharacterized membrane proteins. We foresee that extending these methods to highly valued membrane proteins (e.g., carbohydrate and putative drug transporters) mined from anaerobic fungi and other understudied microorganisms will accelerate strain engineering for a number of industrial applications.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.mec.2019.e00091>.

#### Author Contributions

M.A.O., S.S., and J.I.Y. designed the research, analyzed data, and wrote the manuscript. S.S., J.I.Y., and D.Y. performed experiments.

#### Competing interests

The authors declare no competing interests.

## Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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