

Generalizing a hybrid synthetic promoter approach in *Yarrowia lipolytica*

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Abstract Both varied and strong promoters are essential for metabolic and pathway engineering applications in any host organism. To enable this capacity, here we demonstrate a generalizable method for the de novo construction of strong, synthetic hybrid promoter libraries. Specifically, we demonstrate how promoter truncation and fragment dissection analysis can be utilized to identify both novel upstream activating sequences (UAS) and core promoters—the two components required to generate hybrid promoters. As a base case, the native TEF promoter in *Yarrowia lipolytica* was examined to identify putative UAS elements that serve as modular synthetic transcriptional activators. Resulting synthetic promoters containing a core promoter region activated by between one and twelve tandem repeats of the newly isolated, 230 nucleotide UAS_{TEF#2} element showed promoter strengths 3- to 4.5-fold times the native TEF promoter. Further analysis through transcription factor binding site abrogation revealed the GCR1p binding site to be necessary for complete UAS_{TEF#2} function. These various promoters were tested for function in a variety of carbon sources. Finally, by combining disparate UAS elements (in this case, UAS_{TEF} and UAS1B), we developed a high-

strength promoter with for *Y. lipolytica* with an expression level of nearly sevenfold higher than that of the strong, constitutive TEF promoter. Thus, the general strategy described here enables the efficient, de novo construction of synthetic promoters to both increase native expression capacity and to produce libraries for tunable gene expression.

Keywords Hybrid promoter · Promoter engineering · *Yarrowia lipolytica* · TEF

Introduction

High, tunable levels of gene expression are necessary for metabolic and pathway engineering applications in model host organisms. However, newly isolated or poorly understood organisms often lack well-characterized promoters required for high transcription rates and tunable gene expression. Prior attempts to modulate gene expression include altering endogenous promoter strength through point mutations (Alper et al. 2005; Nevoigt et al. 2006), tuning intergenic regions in operons (Pfleger et al. 2006), and saturation mutagenesis of non-conserved promoter spacer regions (Jensen and Hammer 1998; Jeppsson et al. 2003; Rud et al. 2006). While successful at generating large ranges in relative expression, these methods are quite limited by their tendency to decrease gene expression levels below the original starting point and thus rely on a strong, well-defined starting promoter. To overcome these limitations, we have recently demonstrated the utility of a synthetic hybrid promoter engineering approach in the model yeast *Saccharomyces cerevisiae* and in the oleaginous yeast *Yarrowia lipolytica* (Blazeck et al. 2011, 2012). Here we seek to generalize this approach through the de novo isolation and evaluation of a novel UAS element and, in doing so, create one of the strongest known promoters for *Y. lipolytica*.

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Y. lipolytica is an excellent example of a unique host with intriguing capabilities whose applications, such as native or heterologous protein excretion, were until recently limited by a lack of strong characterized promoters (Barth and Gaillardin 1997; Beopoulos et al. 2009; Madzak et al. 2004; Muller et al. 1998; Nicaud et al. 2002). *Y. lipolytica*'s transcriptional machinery is amenable to a hybrid promoter approach (Blazeck et al. 2011; Madzak et al. 2000), and simple genetic tools are readily available (Chen et al. 1992; Davidow et al. 1985; Fournier et al. 1993; Juretzek et al. 2001; Ledall et al. 1994; Matsuoka et al. 1993; Vanheerikhuisen et al. 1985; Vernis et al. 1997, 2001).

Synthetic hybrid promoters consist of a core promoter region fused to a single upstream activating sequence (UAS) or multiple tandem UAS repeats that augment and modulate promoter function (Blazeck et al. 2011; Deboer et al. 1983; Madzak et al. 2000; Mukai et al. 1992; Rosenberg and Tekamp-olson 1992). These upstream activation regions help localize trans-acting regulatory elements (transcription factors) to increase the transcriptional activity of the core promoter region. Thus, the UAS and core promoter regions act as modular synthetic parts that can be combined to produce a strong, functional UAS-core promoter chimera. In this manner, promoter strength can be elevated by either utilizing a stronger core promoter or by increasing the number of UAS repeats (Blazeck et al. 2011, 2012). All prior hybrid promoter engineering efforts in the oleaginous yeast *Y. lipolytica* have utilized the same 105 basepair upstream activating sequence named UAS1B as the sole UAS element. UAS1B was originally isolated through a functional dissection of the strong, tightly regulated XPR2 native promoter and showed promising constitutive transcriptional activation abilities (Blanchin-roland et al. 1994; Madzak et al. 1999, 2000). Multiple tandem repeats of this UAS element adjacent to a core minimal LEU2 promoter or core TEF-based promoters resulted in significant transcriptional activation (Blazeck et al. 2011; Madzak et al. 2000).

The full flexibility of hybrid promoter generation has been limited by the reliance on only well-established upstream activating regions—a building block likely unidentified in poorly understood hosts. To circumvent this limitation, we describe here the methodology for the isolation, characterization, and incorporation of novel UAS elements into strong, tunable hybrid promoter libraries in *Y. lipolytica*. In particular, we demonstrate the modular transcriptional activation capacity of a 257-basepair upstream region in the *Y. lipolytica* TEF promoter (dubbed UAS_{TEF}). A full dissection of UAS_{TEF} element yielded a fully active, 230 bp UAS, element, dubbed UAS_{TEF}#2. Only six tandem repeats are necessary for saturation of promoter enhancement using this UAS, and a mutational analysis implicates the GCR1p transcription factor binding site to be essential for full UAS function. Kinetic time course experiments reveal that TEF and UAS_{TEF}#2-based

promoters are fully active after 2 days growth before beginning to lose transcriptional activity. Furthermore, characterization in alternative carbon sources demonstrates that these promoters are constitutively active in a variety of conditions. Thus, this work establishes a generalizable approach of hybrid promoter engineering through de novo isolation of novel UAS elements and incorporation into strong, tunable promoter libraries.

Materials and methods

Strains and media

Escherichia coli strain DH10B was used for all cloning and plasmid propagation. DH10B was grown at 37 °C with constant shaking in LB Media Broth (Teknova) supplemented with 50 µg/ml ampicillin for plasmid propagation. *Y. lipolytica* strain PO1f (ATCC # MYA-2613), a leucine and uracil auxotroph devoid of any secreted protease activity (Madzak et al. 2000), was used for all experiments. *Y. lipolytica* PO1f containing plasmid was routinely cultivated at 30 °C with constant agitation in YSC-LEU media consisting of 20 g/L glucose (Fisher Scientific), 0.69 g/L CSM-Leucine (MP Biomedicals), and 0.67 g/L Yeast Nitrogen Base (Becton, Dickinson, and Company). When testing on alternative carbon sources, glucose was replaced by 20 g/L sucrose (Acros Organics), 20 g/L glycerol (Fisher Scientific), or 30 g/L oleic acid (Sigma Aldrich). Solid media for *E. coli* and *Y. lipolytica* was prepared by adding 15 g/L agar (Teknova) to liquid media.

Cloning procedures

All restriction enzymes were purchased from New England Biolabs, and all digestions were performed according to standard protocols. PCR reactions were set up with recommended conditions using Phusion high fidelity DNA polymerase (Finnzymes). Ligation reactions were performed overnight at 22 °C using T4 DNA Ligase (Fermentas). Gel extractions were performed using the Fermentas GeneJET extraction kit purchased from Fisher ThermoScientific. *E. coli* minipreps were performed using the Zyppy Plasmid Miniprep Kit (Zymo Research Corporation). Transformation of *E. coli* strains was performed using standard electroporation protocols (Sambrook and Russell 2001). Transformation of *Y. lipolytica* was performed using the Zymogen Frozen EZ Yeast Transformation Kit II (Zymo Research Corporation). Genomic DNA was extracted from *Y. lipolytica* using the Wizard Genomic DNA Purification kit (Promega).

Plasmid construction

Primer sequences can be found in Table S1. All *Y. lipolytica* plasmids were centromeric, replicative vectors derived from

plasmid pSI16-Cen1-1(227) (Yamane et al. 2008), which was modified to include a multicloning site, a hrGFP green fluorescent reporter gene (pIRES-hrGFP, Agilent), and a *cycl1* terminator (Mumberg et al. 1995) to create plasmid pMCS-hrGFP. A TEF promoter (Damude et al. 2006; Muller et al. 1998) was added to pMCS-hrGFP to form pMCS-TEF-hrGFP. Vectors pMCS-HrGFP, pMCS-TEF-HrGFP, pUC-UAS1B₁₆-TEF, pUC-UAS1B₈-TEF, pMCS-TEF(136)-hrGFP, pMCS-TEF-LacZ, and pUC-UAS1B₂-Leum have been described previously (Blazek et al. 2011). Unless stated otherwise, all PCRs utilized pMCS-TEF-hrGFP as template. All plasmids were sequenced confirmed.

Construction of UAS_{TEF(n)}-TEF and UAS_{TEF(n)}-Leum hrGFP expression cassettes A UAS_{TEF} fragment amplified by primers JB438/439 was *SphI/HindIII* digested and inserted in place of the UAS1B₁₆ fragment in pUC-UAS1B₁₆-TEF to form pUC-UAS_{TEF(1)}-TEF. A second UAS_{TEF} fragment (primers JB440/437) was inserted into pUC-UAS_{TEF(1)}-TEF with *BstBI/SphI* to form pUC-UAS_{TEF(2)}-TEF. UAS_{TEF(1)}-TEF and UAS_{TEF(2)}-TEF promoters were gel extracted and inserted into pMCS-hrGFP with *BstBI/AscI* to create pMCS-UAS_{TEF(1)}-TEF-hrGFP and pMCS-UAS_{TEF(2)}-TEF-hrGFP, respectively. A final UAS_{TEF} fragment (primers JB442/443) was inserted into pMCS-UAS_{TEF(2)}-TEF-hrGFP with *XmaI/BstBI* to form pMCS-UAS_{TEF(3)}-TEF-hrGFP, completing UAS_{TEF(n)}-TEF expression cassette construction (Fig. 1a).

An *EcoRI/SphI* digested UAS_{TEF} fragment amplified by primers JB436/437 was inserted in place of the UAS1B₂ fragment in plasmid pUC-UAS1B₂-Leum to create plasmid pUC-UAS_{TEF(1)}-Leum. A *BstBI/EcoRI* digested UAS_{TEF} fragment (primers JB440/441) was inserted into pUC-UAS_{TEF(1)}-Leum to create pUC-UAS_{TEF(2)}-Leum. UAS_{TEF(1)}-Leum and UAS_{TEF(2)}-Leum promoters were inserted into pMCS-hrGFP with *BstBI/AscI* to create pMCS-UAS_{TEF(1)}-Leum-hrGFP and pMCS-UAS_{TEF(2)}-Leum-hrGFP, respectively. A final UAS_{TEF} fragment (primers JB442/443) was inserted into pMCS-UAS_{TEF(2)}-Leum-hrGFP with *XmaI/BstBI* to form pMCS-UAS_{TEF(3)}-Leum-hrGFP, completing UAS_{TEF(n)}-Leum expression cassette construction.

Construction of the UAS_{TEF(2)}-UAS1B₈-TEF-hrGFP expression cassette A *BstBI/EcoRI* digested UAS_{TEF} fragment amplified with primers JB440/441 was inserted 5' of the UAS1B₈ region of pUC-UAS1B₈-TEF to form pUC-UAS_{TEF}-UAS1B₈-TEF. Promoter UAS_{TEF}-UAS1B₈-TEF was extracted with *BstBI/AscI* and inserted into pMCS-hrGFP, and a final 5' UAS_{TEF} fragment was added with *XmaI/BstBI* to create pMCS-UAS_{TEF(2)}-UAS1B₈-TEF-hrGFP (Fig. 1b).

Dissection of the TEF upstream region Twenty-two overlapping fragments spanning the UAS_{TEF} region were inserted 5' of the TEF(136) minimal promoter in plasmid pMCS-TEF(136)-hrGFP with *XmaI/BstBI* digests to form plasmids pMCS-UAS_{TEF#1}-TEF(136)-hrGFP through pMCS-UAS_{TEF#22}-TEF(136)-hrGFP. Primer pairs JB442/443, JB442/508, JB442/509, JB442/510, and JB442/511 amplified fragments UAS_{TEF#1} through UAS_{TEF#5}. JB503/443, JB503/507, JB503/508, JB503/510, and JB503/511 amplified fragments UAS_{TEF#6} through UAS_{TEF#10}. JB504/443, JB504/509, and JB504/511 amplified fragments UAS_{TEF#11} through UAS_{TEF#13}. JB505/507, JB505/508, JB505/510, and JB505/511 amplified fragments UAS_{TEF#14} through UAS_{TEF#17}. Finally, JB506/443, JB506/507, JB506/508, JB506/510, and JB506/511 amplified fragments UAS_{TEF#18} through UAS_{TEF#22} (Fig. 4a).

Construction of library of UAS_{TEF#2(n)}-TEF(136) hrGFP expression cassettes An initial UAS_{TEF#2} region amplified by primers JB544/537 was inserted into plasmid pUC-UAS1B₈-TEF(136) in place of UAS1B₈ with *EcoRI/HindIII* to form plasmid pUC-UAS_{TEF#2(1)}-TEF(136). Second (primers JB546/539) and third UAS_{TEF#2} (JB548/541) elements were inserted into pUC-UAS_{TEF#2(1)}-TEF(136) with *EcoRI/BamHI* and then *EcoRI/XbaI* digests to create pUC-UAS_{TEF#2(2)}-TEF(136) and then pUC-UAS_{TEF#2(3)}-TEF(136), respectively. To enable plasmid construction, a multicloning site annealed together from JB630/631 was inserted into p416-MCS-yECitrine (Blazek et al. 2012) to create plasmid pTMCS. A 737-bp fragment containing three tandem UAS_{TEF#2} elements in series amplified from pUC-UAS_{TEF#2(3)}-TEF(136) plasmid DNA with primers JB620/621 was inserted twice sequentially into pTMCS, first using *SaII-HF/HindIII* then *SphI-HF/EcoRI-HF* to create plasmids pTMCS-UAS_{TEF#2(3)} and pTMCS-UAS_{TEF#2(6)}. This same 737-bp fragment, digested with *SphI/EcoRI*, was inserted into the three pUC-UAS_{TEF#2(1, 2, and 3)}-TEF(136) plasmids to create plasmids pUC-UAS_{TEF#2(4, 5, and 6)}-TEF(136). The six promoters—UAS_{TEF#2(1)}-TEF(136) through UAS_{TEF#2(6)}-TEF(136)—were *BstBI/AscI*-extracted and inserted into pMCS-hrGFP to create pMCS-UAS_{TEF#2(1)}-TEF(136)-hrGFP through pMCS-UAS_{TEF#2(6)}-TEF(136)-hrGFP. Three or six UAS_{TEF#2} tandem repeats were *BstBI/XmaI* extracted from pTMCS-UAS_{TEF#2(3)} or pTMCS-UAS_{TEF#2(6)} and inserted into pMCS-UAS_{TEF#2(6)}-hrGFP to create pMCS-UAS_{TEF#2(9)}-TEF(136)-hrGFP and pMCS-UAS_{TEF#2(12)}-TEF(136)-hrGFP, completing UAS_{TEF#2(n)}-TEF(136)-hrGFP library construction (Fig. 1c).

Construction of the lacZ expression cassettes The β -galactosidase gene encoded by *E. coli lacZ* (Kalnins et al. 1983) was gel-extracted from pMCS-TEF-lacZ with *AscI/PacI* and inserted in place of hrGFP in certain pMCS-

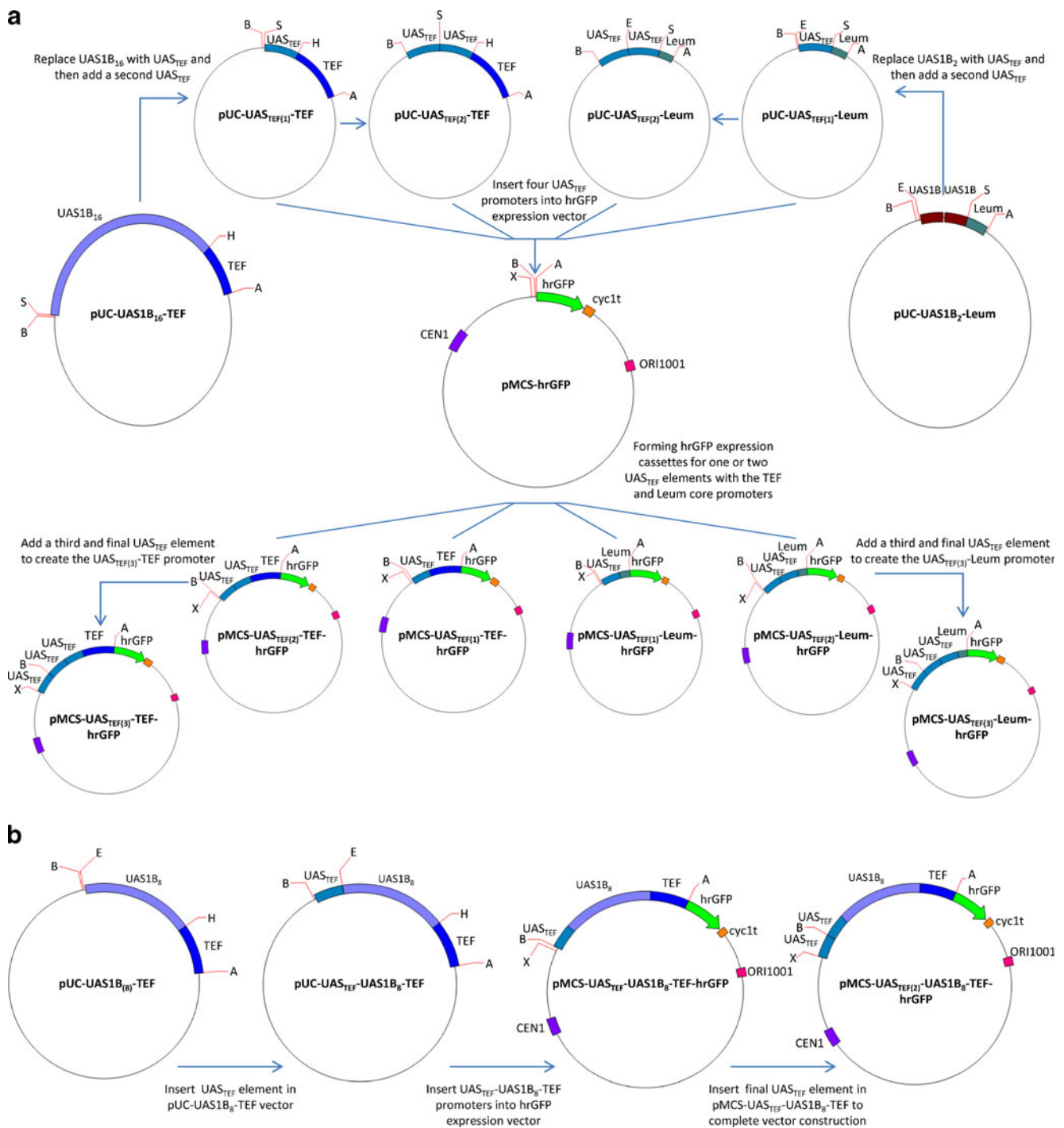


Fig. 1 Construction of plasmids for this study. **a** A schematic picture is provided detailing the construction of UAS_{TEF(n)}-TEF and UAS_{TEF(n)}-Leum promoter hrGFP fluorescence cassettes, **b** the construction of the UAS_{TEF(2)}-UAS1B₈-TEF-hrGFP expression cassette, **c** and the

construction of a library of UAS_{TEF#2} hrGFP expression cassettes. Restriction enzymes utilized are abbreviated as follows: *A*—*Asc*I, *Ba*—*Bam*HI, *B*—*Bst*BI, *E*—*Eco*RI, *H*—*Hind*III, *Sa*—*Sal*I, *S*—*Sph*I, *Xb*—*Xba*I, *X*—*Xma*I

hrGFP-based plasmid series to generate lacZ expression cassettes.

Construction of mutant UAS_{TEF#2} elements Utilizing plasmid pUC-UAS_{TEF#2(1)}-TEF(136) as DNA template for the

Stratagene Quikchange mutagenesis kit, three putative transcription factor binding sites (TFBSs) were removed from UAS_{TEF#2}. Primers JB682/683 deleted a “tgtgt” motif to abrogate a putative NDT80p TFBS. Primers JB680/681 deleted a “ttaag” motif to remove a putative MCM1 TFBS,

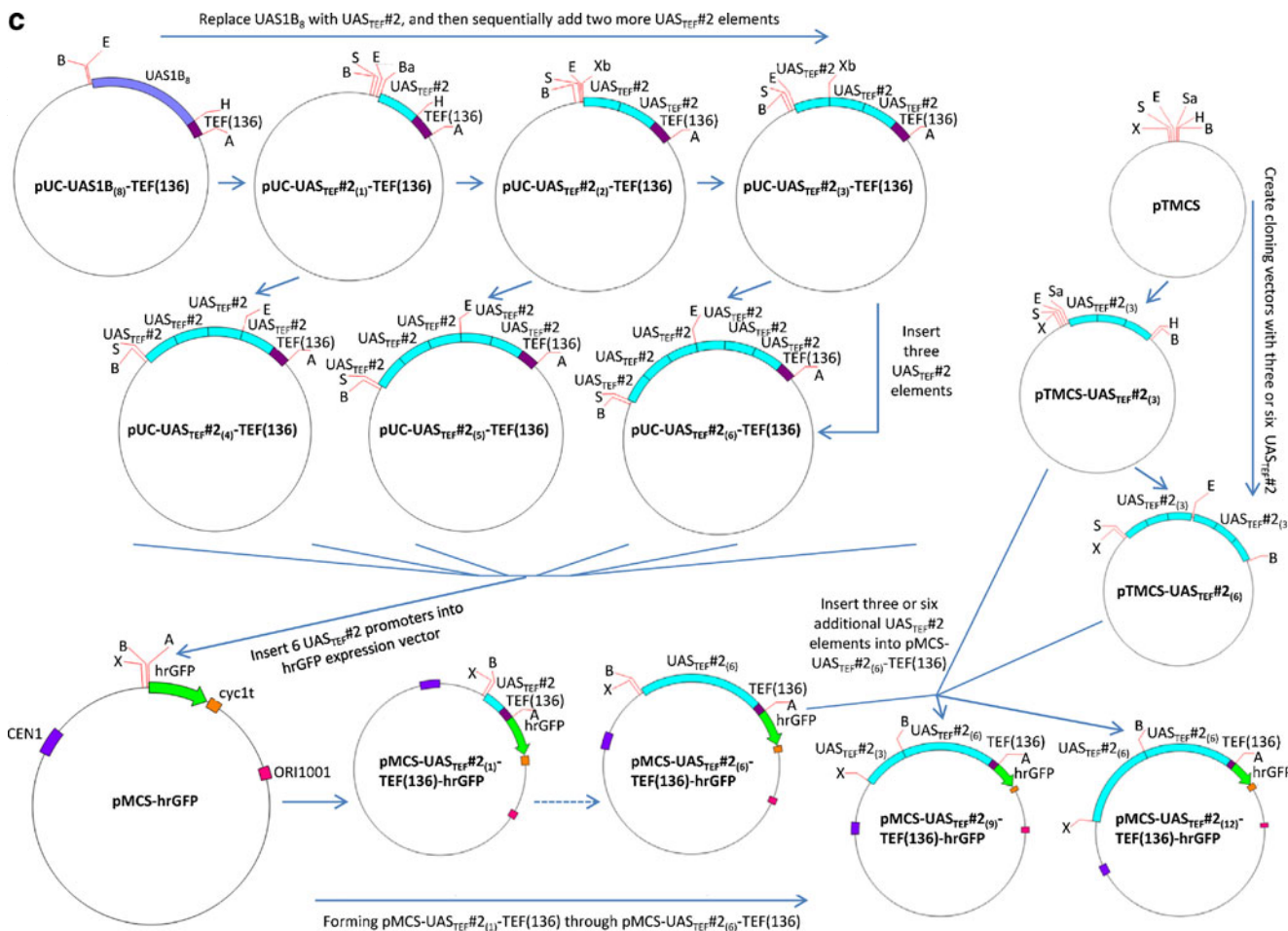


Fig. 1 (continued)

“gccatc” motif to remove a GCRp putative TFBS. Sequential mutagenesis reactions created all combinations of NDT80p, MCM1p, and GCR1p TFBS deletion mutants in the pUC-UAS_{TEF#2(1)}-TEF(136) background. These seven mutants were *Bst*B1/*Asc*I-extracted and inserted into pMCS-hrGFP to complete mutant UAS_{TEF#2} expression cassette construction (Fig. 4c).

Promoter characterization by flow cytometry

The hrGFP green fluorescent protein has been validated as an ideal tool to assess promoter strength in *Y. lipolytica* at the single cell level (Blazcek et al. 2011), and thus was employed to assess relative promoter activity for the majority of results generated in this study. *Y. lipolytica* PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stock (in biological triplicate) in YSC-LEU media for 48 h at 30 °C with shaking, and then normalized to an OD₆₀₀ of 0.01 in 2 ml fresh YSC-LEU and incubated for another 48 h (unless otherwise stated) at 30 °C in a rotary drum (CT-7, New Brunswick Scientific) at speed seven. A time course of fluorescence value showed 48 h to

be an optimal incubation time for high expression levels from native and hybrid promoters (Blazcek et al. 2011). To harvest, the cultures were spun down at 4 °C at 1,000×g for 5 min, washed, and resuspended in 1 ml ice cold water before testing with a FACS Fortessa (BD Biosciences) using the GFP fluorochrome, a voltage of 319, and a 10,000 cell count for hrGFP detection. Samples were kept on ice during the test, and the data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR) to compute mean fluorescence values. Day-to-day variability was mitigated by analyzing all comparable strains on the same day. An average fluorescence and standard deviation were calculated from the mean values of biological replicates.

Promoter characterization through β-galactosidase assay

Y. lipolytica PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stock (in biological triplicate) in YSC-LEU media for 48 h at 30 °C in a rotary drum (CT-7, New Brunswick Scientific) at speed seven, and then normalized to an OD₆₀₀ of 0.01 in 2 ml fresh YSC-LEU and incubated for another 48 h in the same

conditions. The cultures were washed twice and resuspended in 1 ml Z buffer, and their OD₆₀₀ readings were recorded (Gaillardin and Ribet 1987; Miller 1972). β -galactosidase assays were performed as described by Miller (1972) using 25 μ l of chloroform-permeabilized cells, with a reaction time of 17 min.

Kinetic analysis of promoters

Y. lipolytica PO1f strains, transformed with different plasmids, were inoculated directly from glycerol stock (in biological triplicate) in YSC-LEU media for 48 h at 30 °C in a rotary drum (CT-7, New Brunswick Scientific) at speed seven and then normalized to an OD₆₀₀ of 0.01 in 2 ml fresh YSC-LEU and incubated for either 24, 48, 72, or 96 h in the same conditions. Cultures were inoculated such that all cultures (for the 24-, 48-, 72-, and 96-h time points) were harvested and tested via flow cytometry at the same time.

Results

Isolation of a *Y. lipolytica* TEF promoter region UAS

Prior analysis of the 406 basepair full length TEF promoter revealed that the 136 basepair proximal to the ATG start codon, dubbed TEF(136), was unable to drive the expression of an hrGFP reporter gene (Blazek et al. 2011). However, when this core promoter fragment was used in conjunction with a known UAS element, resulting TEF(136)-based hybrid promoters generated high fluorescence levels. These results established TEF(136) as a functional, low-strength core promoter that had been apparently stripped of its native UAS elements (Blazek et al. 2011). Analysis of the TEF promoter revealed that a drastic change in GC content coincided with the end of all putative TATA elements at 149 basepairs upstream of the ATG start codon. Consequently, we postulated that a strong upstream activating sequence responsible for the majority of the TEF promoter's transcriptional activation ability must be located further upstream in the remaining 257 basepairs. We tested this hypothesis by constructing two distinct series of hybrid promoters in which between one and three tandem repeats of a putative 257 basepair TEF upstream activating sequence, dubbed UAS_{TEF}, were fused to a core promoter. Hybrid promoter series UAS_{TEF(n)}-TEF employed the 406 basepair native TEF promoter (TEF) as a core promoter, while series UAS_{TEF(n)}-Leum employed a minimal core LEU2 promoter (Leum). Flow cytometry analysis of the UAS_{TEF(n)}-TEF and UAS_{TEF(n)}-Leum hybrid promoters revealed strong expression enhancement by tandem UAS_{TEF} elements (Fig. 2). The enhancement of fluorescence levels

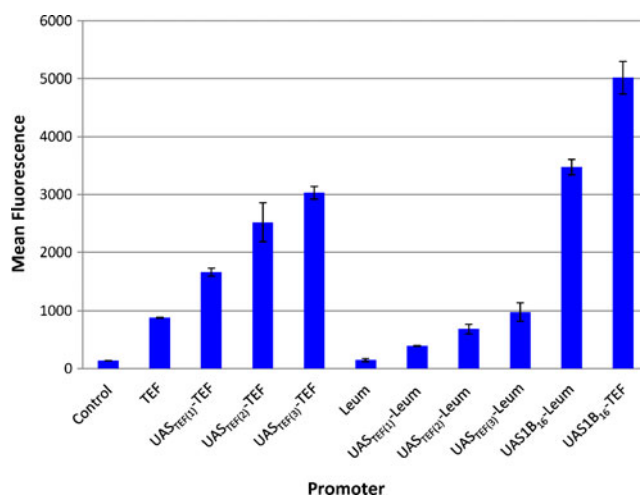


Fig. 2 Functional testing of the novel UAS_{TEF} element. Relative fluorescence values indicate that the UAS_{TEF} element functions as a strong modular upstream activating sequence, linearly increasing expression capacity of both the TEF native promoter ($r^2=0.9899$) and the Leum minimal promoter ($r^2=0.9983$). Three tandem repeats of the UAS_{TEF} element elevated protein expression to more than threefold base levels for the TEF promoter and sixfold for the Leum promoter. Final expression capacity of the UAS_{TEF(3)}-TEF promoter approached UAS1B₁₆-TEF and UAS1B₁₆-Leum levels, two of the strongest previously characterized hybrid promoter in *Y. lipolytica*. Error bars represent the standard deviation of measurements between biological triplicates

were strongly linearly correlated to the number of UAS_{TEF} copies ($r^2=0.9899$ for TEF core and $r^2=0.9983$ for Leum core— r^2 values calculated using a linear regression in Microsoft Excel), demonstrating the modularity and functional additivity of this novel UAS. Net promoter strength strongly depended on core promoter choice, as hybrid promoters employing the TEF core were more than threefold stronger than Leum-based counterparts. The maximum expression amplification imparted to each core promoter differed as well. In this vein, three UAS_{TEF} tandem repeats generated a 6.45-fold amplification of expression when fused to the Leum core promoter (compared to basal Leum), while only a 3.44-fold increase emerged in the corresponding TEF-based constructs. These results echo our previous findings that amplification levels were highest with more minimal core promoter constructs.

Investigating the effect of incorporating disparate UAS sequences

We next investigated the impact of combining disparate UAS elements. The UAS_{TEF} element proximal to the core promoter in promoter UAS_{TEF(3)}-TEF was replaced by an UAS1B₈ fragment (Blazek et al. 2011) to form hybrid promoter UAS_{TEF(2)}-UAS1B₈-TEF. The mean fluorescence levels generated by UAS_{TEF(2)}-UAS1B₈-TEF are at least as

high as the strongest promoters yet described in *Y. lipolytica* (Blazek et al. 2011), advocating future work assessing potential benefits of incorporating multiple, distinct UAS elements into hybrid promoters (Fig. 3).

Functional dissection analysis of the UAS_{TEF} element through promoter truncation and transcription factor binding site removal

With the 257-bp UAS_{TEF} established as a modular, synthetic transcriptional amplifier, we sought to dissect its genetic sequence in search of a more minimal, compact UAS element. Twenty-two unique but overlapping fragments spanning the UAS_{TEF} sequence were PCR-amplified to create a UAS_{TEF}-based truncation library (Fig. 4a). Putative UAS library elements were individually fused to the minimal TEF (136) core promoter to form promoters UAS_{TEF}#1-TEF (136) through UAS_{TEF}#22-TEF(136). The minimal TEF (136) core promoter was selected for hybrid promoter construction to allow for the highest sensitivity in measuring UAS activity. The newly constructed hybrid promoter series was tested with hrGFP-based flow cytometry analysis. The fluorescence data reveal that the majority of truncated UAS_{TEF} fragments continue to retain at least modest UAS activity (Fig. 4a, b). This result suggests that nearly all of these fragments can serve to create functional, synthetic hybrid

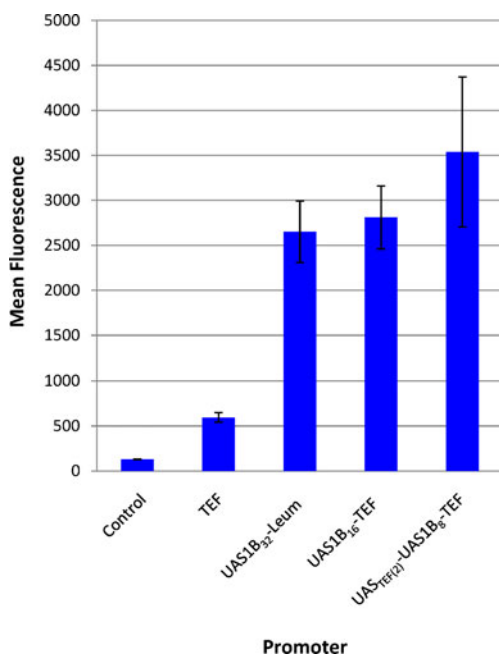


Fig. 3 Combining the novel UAS_{TEF} with the UAS1B element. Relative fluorescence values indicate that the UAS_{TEF} (2)-UAS1B₈-TEF promoter surpasses in strength the previously characterized UAS1B₁₆-TEF and UAS1B₃₂-Leum promoters, the strongest promoters constructed in *Y. lipolytica*. Error bars represent the standard deviation of measurements between biological triplicates

promoters. However, hybrid promoter strength showed a pronounced correlation to UAS_{TEF} fragment length, providing evidence that the majority of the original 257 bp UAS_{TEF} sequence is required for full activity (Fig. 4b). UAS_{TEF}#2 displayed the highest activating capacity of the UAS_{TEF} truncations tested, elevating the strength of the TEF(136) core promoter to levels 1.5-fold times the native TEF promoter (Fig. 4a). Thus, we selected UAS_{TEF}#2 for further hybrid promoter library construction and for a more rigorous examination.

The majority of the UAS_{TEF}#2 element is necessary for full UAS activity, precluding a more thorough characterization through truncation analyses. Thus, we characterized UAS_{TEF}#2 through the systematic removal of three putative transcription factor binding sites predicted within this sequence. In our truncation analysis, we observed that removing 27 basepair at the 5' end of UAS_{TEF} (−406 to −386 deletion) tended to decrease UAS strength, but further truncation had little effect. We also observed that the removal of 27 basepairs from the 3' end of the original UAS_{TEF} (−149 to −176 deletion) often increased UAS strength, but further 3' truncation (−176 to −196/−216/−236 deletion) always decreased UAS strength (Fig. 4a, c). Thus, we concluded the existence of an upstream repressive element (URS) between basepairs −149 to −176 and enhancing UAS elements in the remaining truncated regions (−406 to −386 and −176 to −196/−216/−236 deletion).

An analysis of the native TEF and the UAS_{TEF}#2-TEF (136) promoters at the sequence level in the yeast promoter database (SCPD) (Zhu and Zhang 1999) revealed that consensus binding sites for yeast transcription factors MCM1p and PUT3p are lost in the 27 basepair truncation. Further sequence analysis of the UAS_{TEF}#2 element also highlighted consensus binding sites for transcription factors NDT80p at position −386, for GCR1p at position −186, and for a separate MCM1p at position −257 (Fig. 4c). We hypothesized that the GCR1p and NDT80p binding sites grant UAS activity, while this second native MCM1p binding site reduces UAS activity. To test this hypothesis, we employed site-directed mutagenesis to delete the NDT80p, GCR1p, and MCM1p binding sites from the UAS_{TEF}#2-TEF(136) promoter (Fig. 4c), and tested and compared binding site deletion mutants with hrGFP-based flow cytometry. As expected, removal of the NDT80p and GCR1p bindings sites reduced UAS_{TEF}#2-TEF(136) promoter strength, by 15 % and 31 %, respectively (Fig. 4d). Combinatorial deletion of NDT80p and GCR1p binding sites only slightly further decreased promoter strength, revealing the GCR1p binding site as the predominate site responsible for UAS_{TEF} #2 function. Deletion of the MCM1p binding site had no effect on UAS_{TEF}#2-TEF(136) promoter strength (Fig. 4d). Thus, the GCR1p binding site

motif is essential for UAS capability, while URS activity is not conferred by the MCM1p binding site.

Utilizing the hybrid promoter approach to construct a novel tandem UAS series

Above, we have demonstrated how UAS elements can be deduced and mechanistically studied through promoter truncation analysis. To complete the generic hybrid promoter construction process, we utilized one, two, three, four, five, six, nine, or twelve tandem repeats of the newly isolated UAS_{TEF#2} sequence to enhance expression of the minimal TEF(136) core promoter. These new UAS_{TEF#2} based promoters were tested and compared to the native TEF promoter, amongst other controls, via hrGFP fluorescence by flow cytometry. Multiple copies of UAS_{TEF#2} increased hybrid promoter strength for up to six tandem repeats, at which point expression enhancement from added UAS_{TEF #2} elements had been saturated (Fig. 5a). Ultimately, UAS_{TEF #2}-TEF(136) hybrids yielded expression levels 3.5-fold higher than the native, full length TEF promoter starting point (Fig. 5a). Expression cassettes containing tandem UAS_{TEF #2} copies without the TEF(136) promoter generated no expression above background levels. To further demonstrate the utility of the UAS_{TEF#2}-TEF(136) series, we utilized the β -galactosidase gene as alternate reporter protein. Once again, UAS_{TEF#2}-mediated enhancement increased promoter activity, producing a final expression enhancement to nearly twice that of the native TEF using this marker (Fig. 5b).

Kinetic analysis of hybrid promoters and effect of media formulation

Previous analysis of the UAS1B element (the only other known upstream activating sequence in *Y. lipolytica*) revealed expression profiles dependent on both time course and media formulation (Madzak et al. 2000, 2004). Thus, we sought to further characterize our novel UAS_{TEF}-based promoters and compare them to several previously constructed hybrid promoters utilizing a thorough time course kinetic analysis and analyzing the effects of alternate carbon sources on promoter activity.

We analyzed the UAS_{TEF#2(n)}-TEF(136) series, several high expression UAS_{TEF}-truncation hybrids, the novel UAS_{TEF(2)}-UAS1B₈-TEF promoter, and our previously constructed strong hybrids promoters UAS1B₈-TEF(406), UAS1B₁₆-TEF(406), UAS1B₁₆-Leum, and UAS1B₃₂-Leum (Blazcek et al. 2011) via hrGFP flow cytometry after 1, 2, 3, and 4 days of growth to discern effects of cell phase on promoter activity. UAS_{TEF#2}-TEF(136) promoters demonstrated fairly constitutive activity, peaking in expression levels after two days before decreasing (Fig. 6a). Interestingly, the UAS_{TEF#2(6)}-TEF(136) and UAS_{TEF#2(12)}-TEF

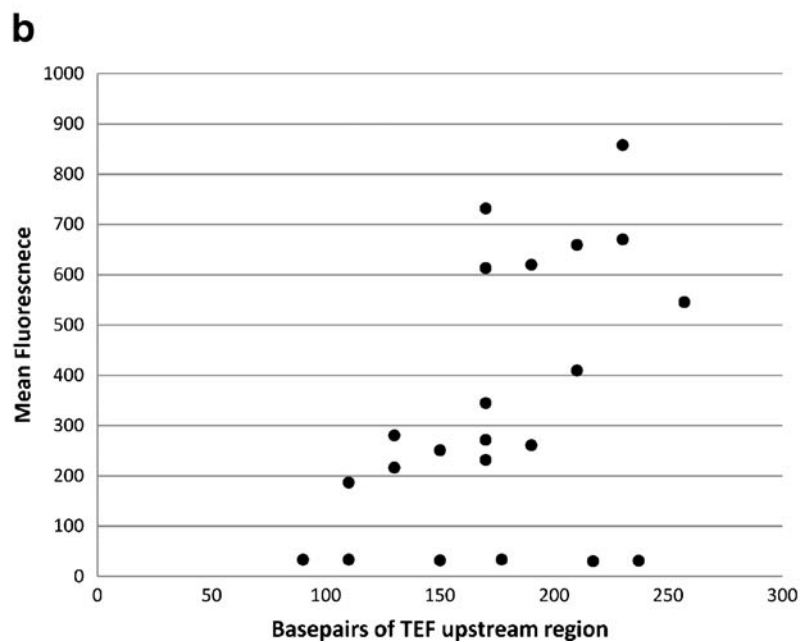
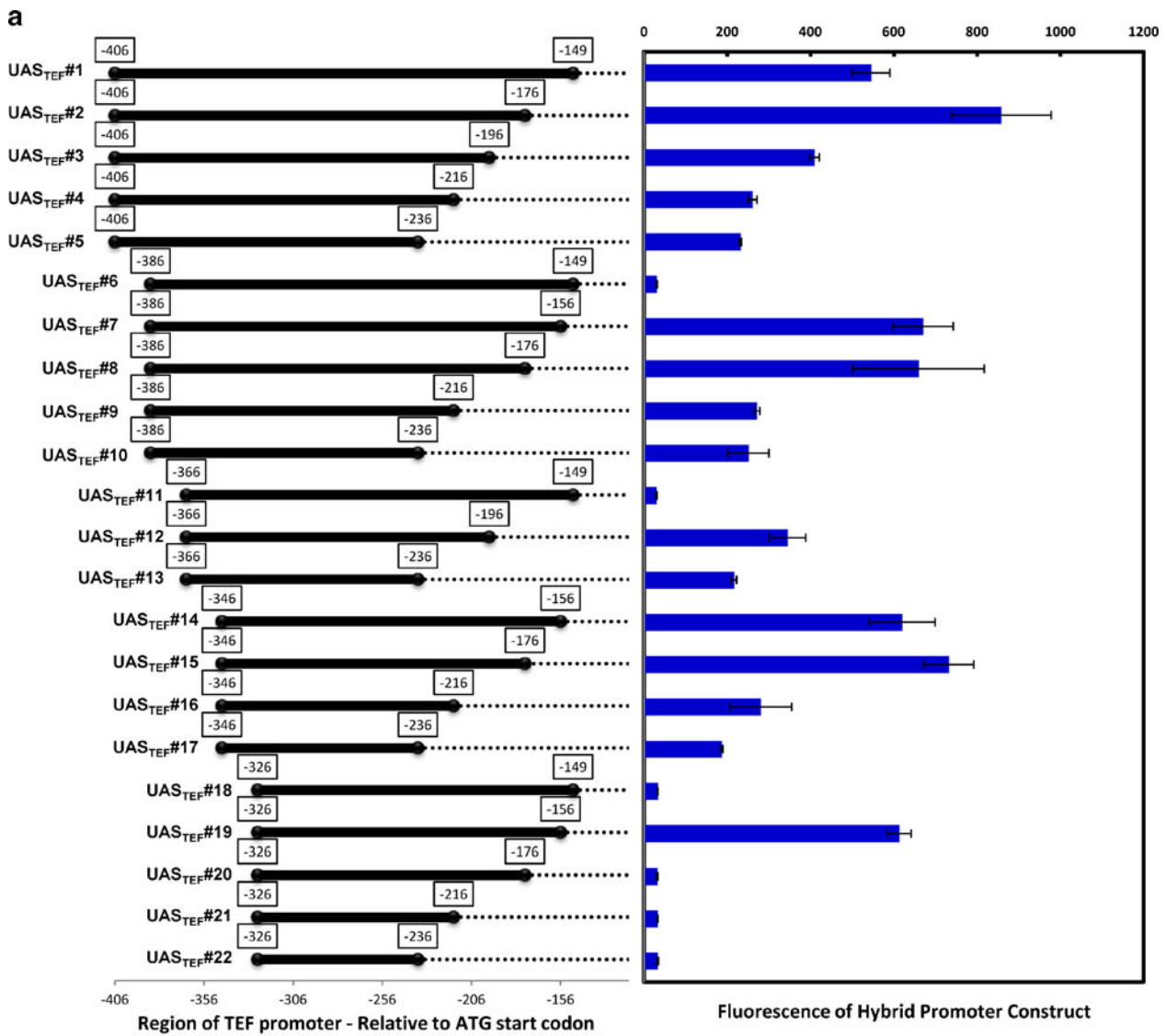
(136) promoter attain nearly identical peak expression level, but the UAS_{TEF#2(12)}-TEF(136) promoter retains full activity much longer. Hybrid promoters based on the UAS_{TEF} truncations displayed similar constitutive expression profiles, with the UAS_{TEF#2}-TEF(136) promoting the highest expression levels (Fig. 6b). While stronger than the UAS_{TEF}-based hybrids, the UAS1B₁₆-Leum and UAS1B₃₂-Leum promoters were not highly activated until the third day of growth, confirming prior observation that the UAS1B element is most active in early stationary phase (Madzak et al. 1999, 2000). The UAS1B₈-TEF(406) and UAS1B₁₆-TEF(406) promoters displayed very high fluorescence levels and were fully expressed after only 2 days. The UAS_{TEF(2)}-UAS1B₈-TEF promoter reached maximal expression capacity quicker than any other tested promoter, further demonstrating the benefit of incorporating multiple, distinct UAS elements into the same hybrid promoter (Fig. 6c).

To analyze the effects of media on promoter expression capacity, we tested many of the hybrid constructs for protein expression when grown in media utilizing glucose, sucrose, glycerol, or oleic acid as the sole carbon source. UAS_{TEF#2}-TEF(136) promoters were minimally impacted by carbon source, with sucrose enabling the highest expression, and glycerol the lowest expression capacity (Fig. 7a). In contrast, promoters containing UAS1B elements were very strongly dependent on media composition, with highest expression on sucrose or oleic acid (Fig. 7b). Thus, hybrid promoters constructed with the newly isolated TEF UAS were more constitutive than previous *Y. lipolytica* hybrid promoters.

Discussion

This study generalizes a synthetic hybrid promoter approach for generating promoter libraries of increasing strength, especially in organisms lacking strong, characterized promoters. Specifically, this study illustrated the isolation and characterization of a novel upstream activating sequence. In

Fig. 4 Dissection of the UAS_{TEF} element. **a** Twenty-two overlapping fragments spanning the UAS_{TEF} sequence were inserted upstream of a TEF(136)-hrGFP expression cassette and tested for transcriptional amplification activity. Relative fluorescence values are shown. **b** Relative fluorescence values are plotted as a function of putative UAS length, with a noticeable correlation between decreasing putative UAS strength and decreasing UAS length. **c** A simplified schematic picture is provided detailing the location of specific consensus transcription factor binding sites within the UAS_{TEF#2} element and the TEF native promoter. **d** Three putative transcription factor binding sites (NDT80p, MCM1p, and GCR1p) were removed individually and combinatorially from the UAS_{TEF#2} element with at UAS_{TEF#2(1)}-TEF(136)-hrGFP expression cassette. Relative fluorescence values are shown. Error bars represent the standard deviation of measurements between biological triplicates



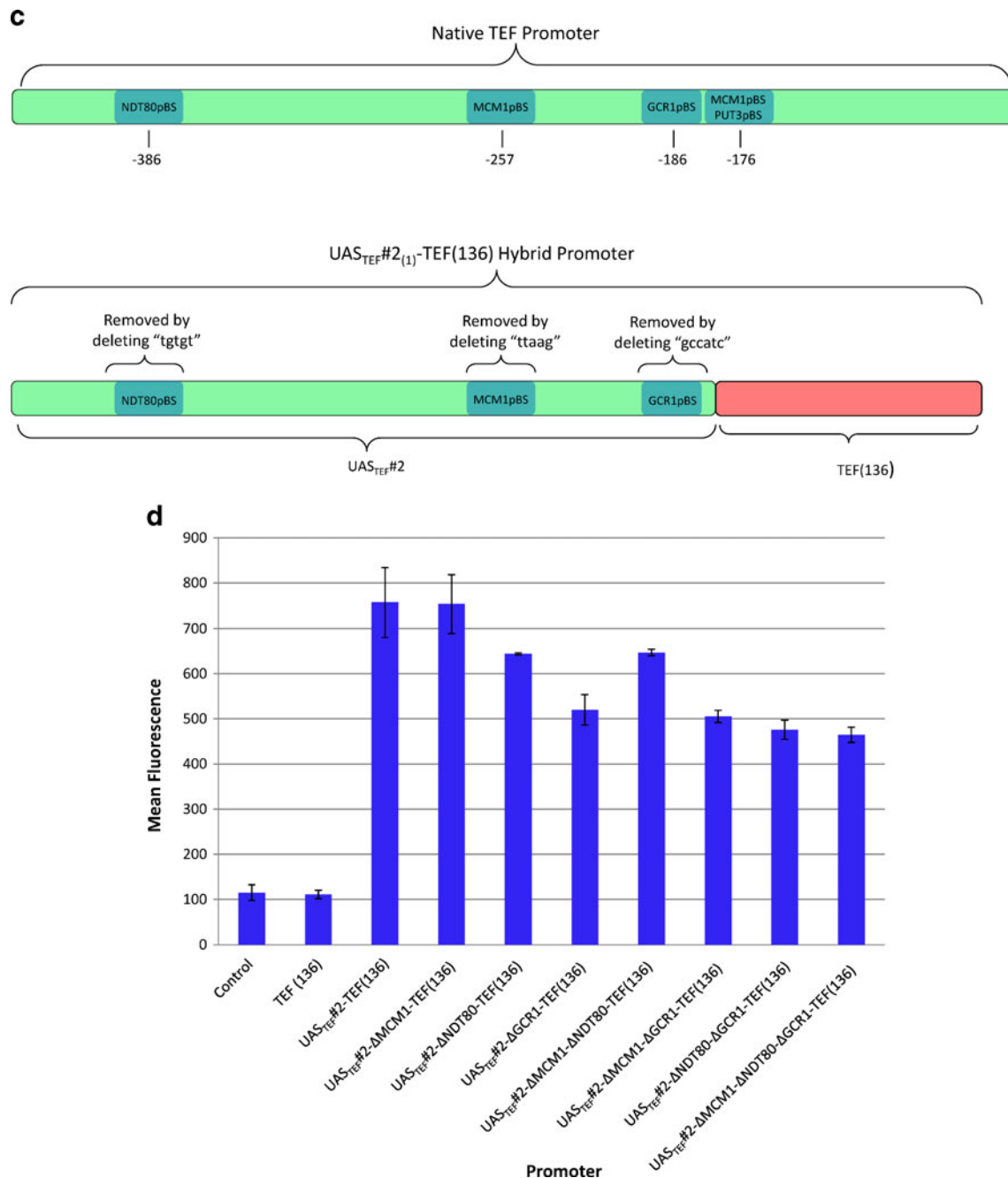


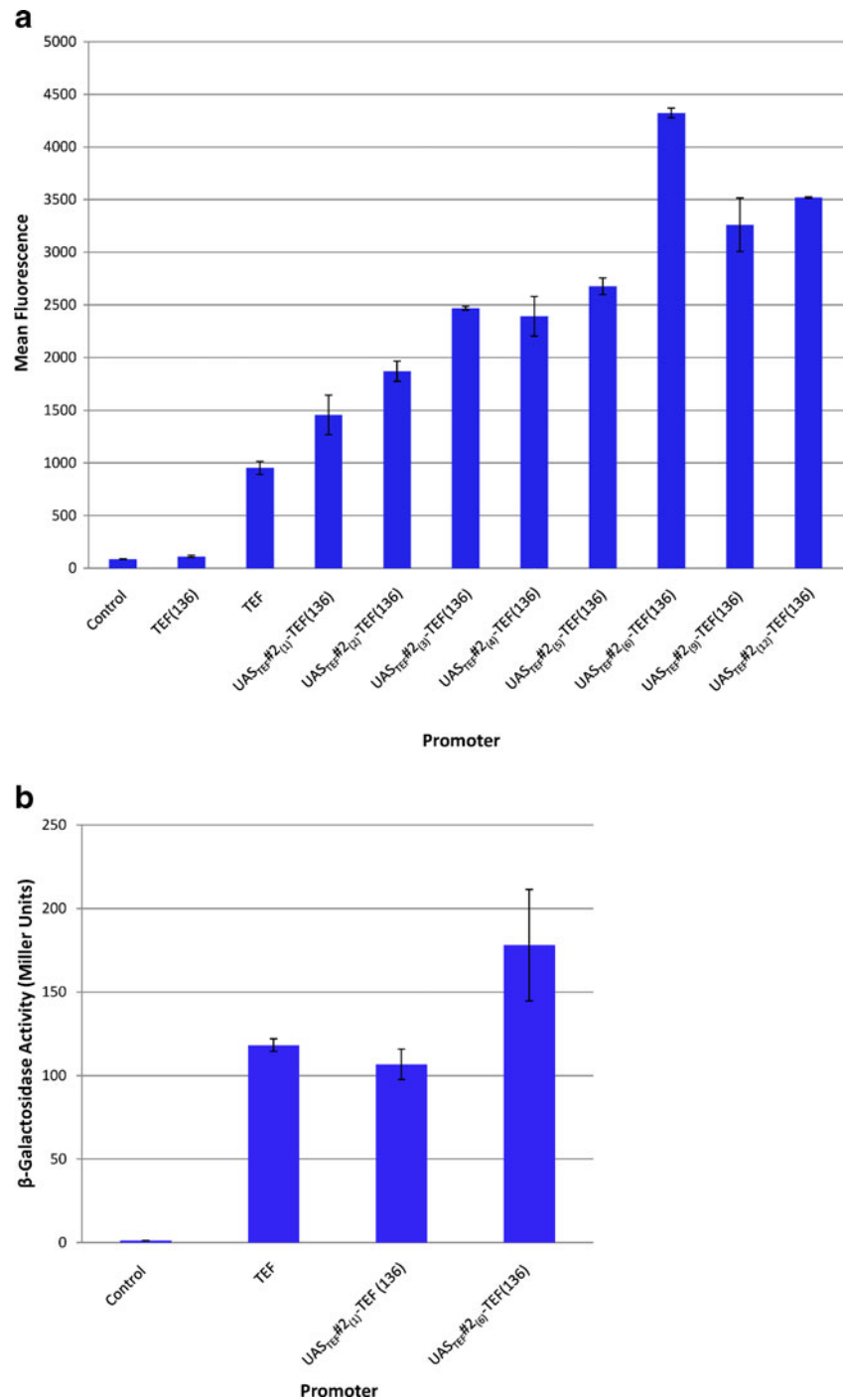
Fig. 4 (continued)

this regard, this approach expands the quantity and quality of parts required for synthetic biology research (Liu et al. 2011; Young and Alper 2010).

In this work, we validated the existence of a strong UAS element upstream of the minimal core TEF(136) sequence, and demonstrated its capacity as a synthetic amplifier when fused to either the minimal leucine promoter or the full native TEF promoter. In this regard, the novel UAS_{TEF} enhancer acts as a modular element that relieves enhancer-limited transcription at core promoter elements. Hybrid promoters using the TEF core were much stronger (at least

threefold) than their Leum-based counterparts, as expected from the difference in their basal core strength. We further demonstrated a rigid linear correlation between promoter strength and the number of UAS_{TEF} enhancer elements. This relationship is in contrast to the Hill-curve dynamics seen with the UAS1B enhancement (Blazcek et al. 2011). This difference suggests that UAS elements have distinct transfer functions of activity. Moreover, both UAS enhancer element and core promoter elements contribute to overall hybrid promoter strength in a logical manner, raising the possibility of rationally designing synthetic hybrid promoters with

Fig. 5 Functional testing of the novel UAS_{TEF}#2 element to complete de novo hybrid promoter construction. **a** Relative fluorescence values indicate that the UAS_{TEF}#2 element functions as an upstream activating sequence, increasing expression capacity of the TEF(136) promoter to levels 3.5-fold higher than the native TEF promoter. Final UAS_{TEF}#2-based promoter strength remained below UAS_{TEF}-based and previously characterized promoters, UAS_{1B₃₂}-Leum and UAS_{1B₁₆}-TEF. **b** Hybrid promoters were tested with a β -galactosidase reporter gene, yielding similar results to the hrGFP assay. *Error bars* represent the standard deviation of measurements between biological triplicates



specified expression strength for a desired metabolic engineering application.

The UAS_{TEF} enhancer element represents a newly isolated upstream activating sequence in *Y. lipolytica*. We conducted a dissection analysis of this UAS_{TEF} to eliminate extraneous (or potentially interfering) DNA sequence as a means of demonstrating a generic methodology for identifying useful UAS elements. While the majority of this 257 bp UAS_{TEF} element was confirmed to be necessary to enable full transcriptional capacity of TEF, the removal of

27 bp from 3' UAS_{TEF} resulted in increased activation potential. Removing this specific portion of DNA alleviates latent repressive activity in the otherwise highly constitutive TEF promoter sequence. A comparison of the native TEF and the UAS_{TEF}#2-TEF(136) revealed that MCM1p and PUT3p binding sites are lost in this truncation, and a deletion analysis of putative transcription factor binding sites revealed that MCM1p binding sites have no effect on TEF promoter activity, implicating the PUT3p binding site as a repressive element. Further mutational analysis highlighted

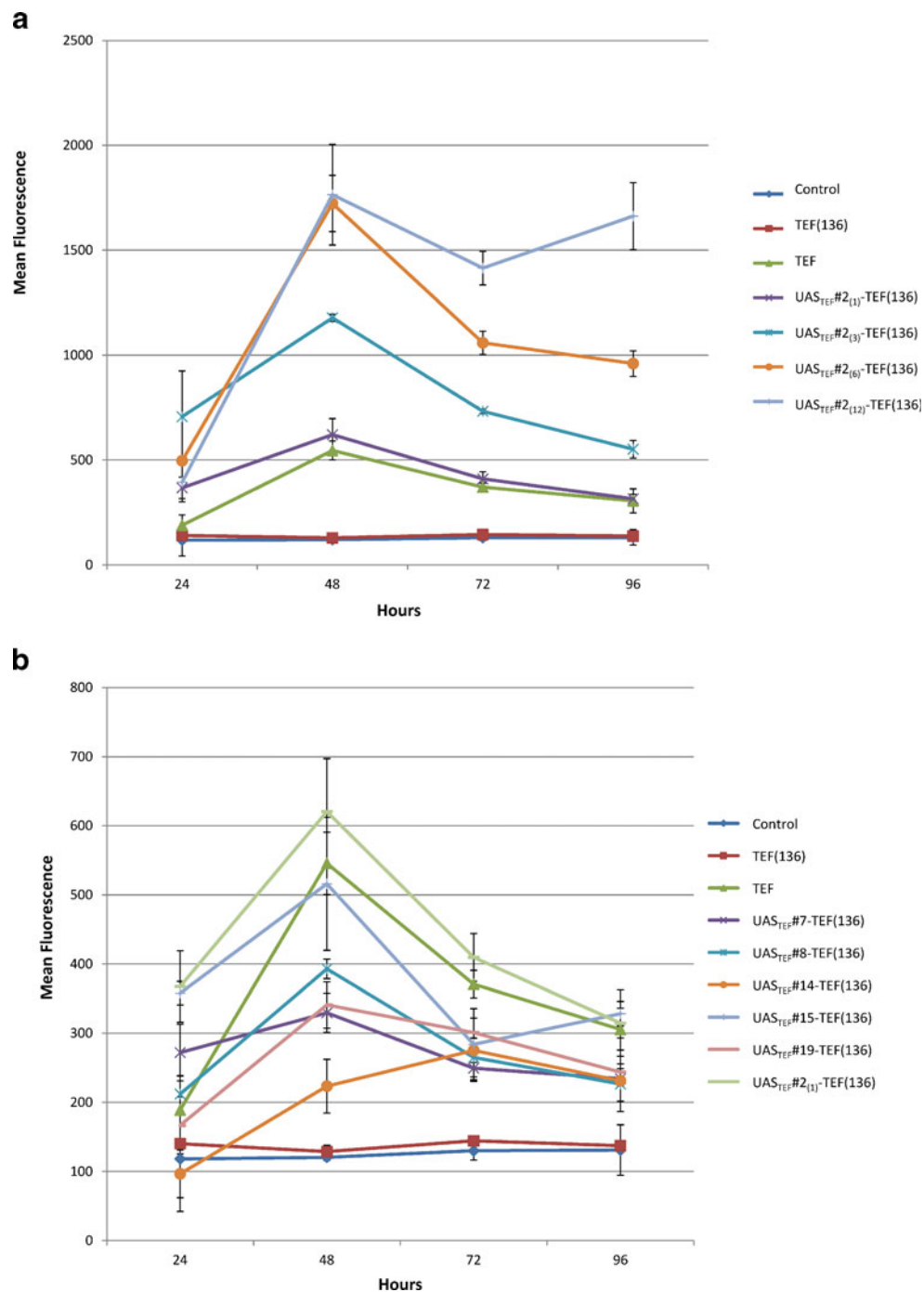


Fig. 6 Kinetic analysis of hybrid promoters. **a** Relative fluorescence values indicate that tandem copies of UAS_{TEF}#2 showed progressive increased in transcription capacity. **b** Various UAS_{TEF} truncation hybrid promoters (from those described in Fig. 3a) displayed similar expression profiles, with the UAS_{TEF}#2-TEF(136) promoting the highest expression levels. **c** UAS1B₁₆-Leum and UAS1B₃₂-Leum promoters were not highly activated until the third day of growth.

UAS1B₈-TEF(406) and UAS1B₁₆-TEF(406) promoters displayed very high fluorescence levels and were fully expressed after only 2 days, and the UAS_{TEF}(2)-UAS1B₈-TEF promoter exhibited the high expression capacity quicker than any other tested promoter. *Error bars* represent the standard deviation of measurements between biological triplicates

the GCR1p binding site as the predominant site responsible for the full transcriptional capacity associated with the TEF_{UAS} element. Thus, by combining a truncation analysis with targeted binding site mutagenesis, we were able to

propose transcription factors localized by the TEF_{UAS} region that both activate and repress transcription and attain a greater understanding of *Y. lipolytica* native promoters. Following this dissection, we fused the strongest UAS_{TEF}

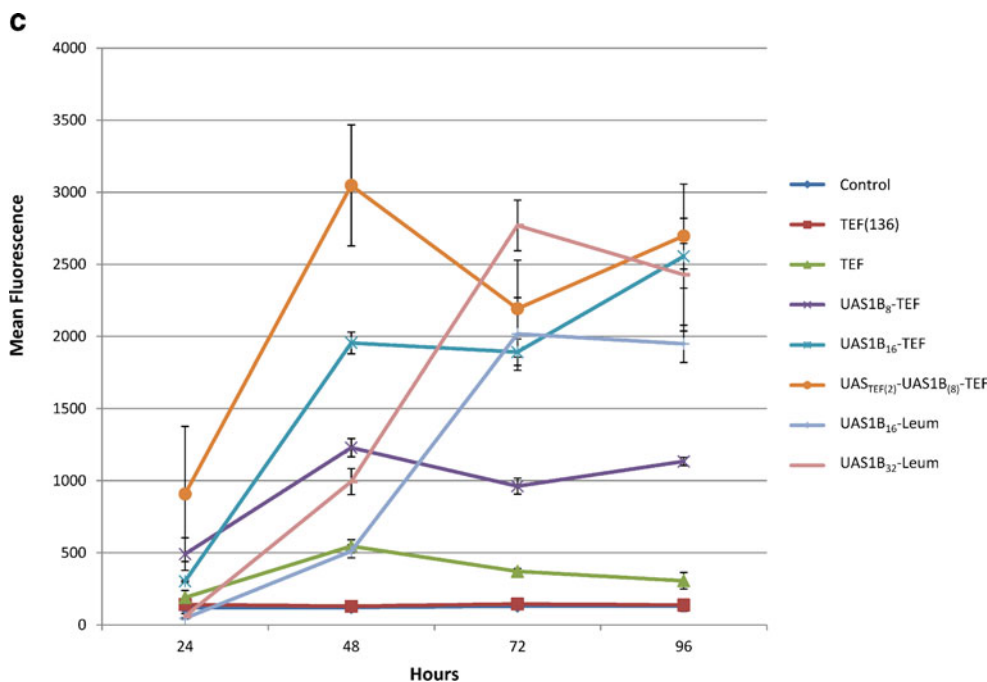


Fig. 6 (continued)

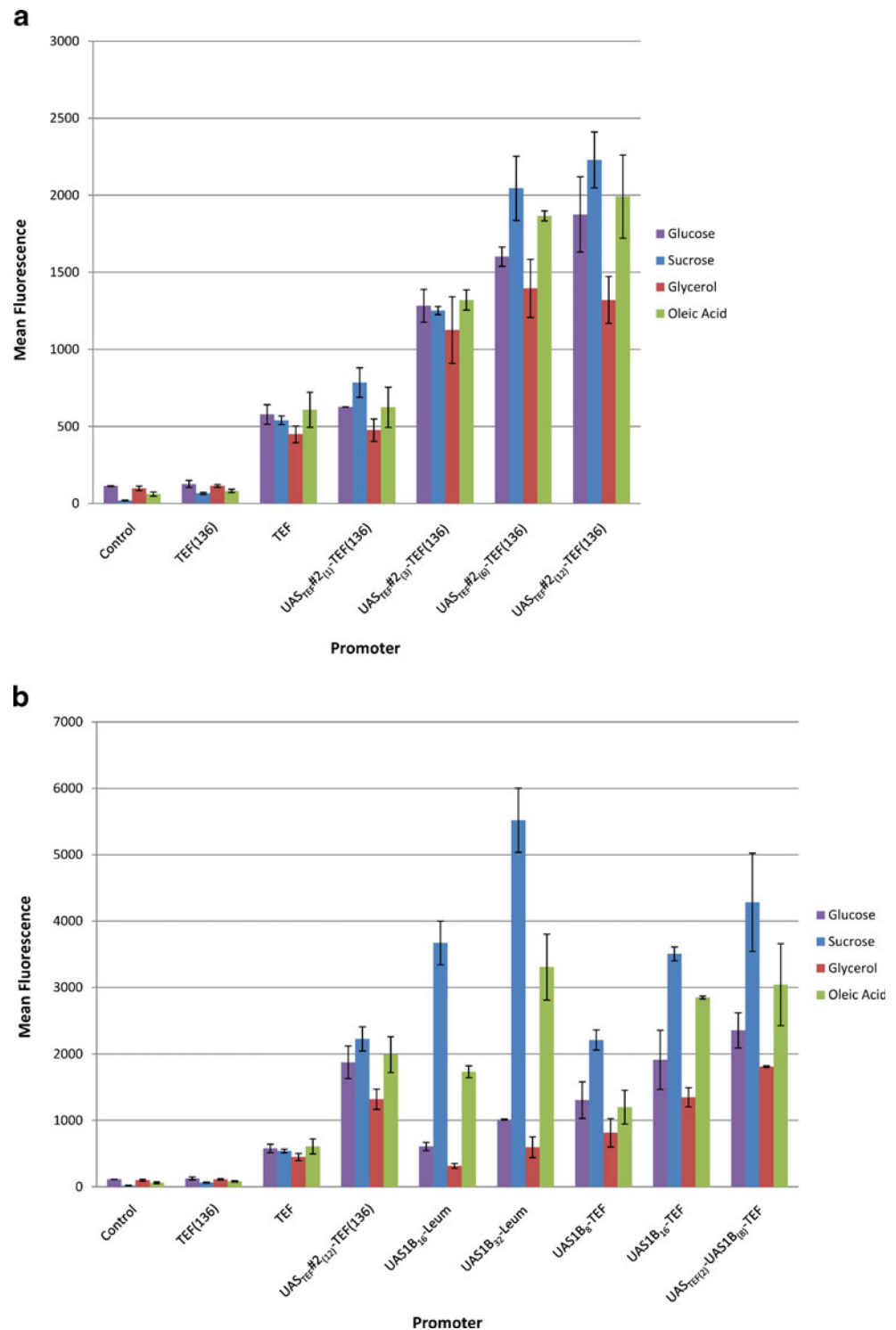
truncation identified, UAS_{TEF#2} (which contained the 3' deletion), in tandem copies to the TEF(136) core promoter. Only six UAS_{TEF#2} elements were necessary to saturate gains in promoter expression capacity from tandem UAS addition, potentially because of a shortage of essential transcription factors utilized by this enhancer element, a metabolic burden observed in high-strength heterologous promoter systems in *S. cerevisiae* (Gorgens et al. 2001). Even so, the maximal fluorescence yielded by the UAS_{TEF#2} library reached levels 3.5-fold that of the native TEF promoter. Thus, UAS elements isolated from native promoters can be utilized to increase available promoter strength within an organism.

As described above, all prior hybrid promoter attempts in *Y. lipolytica* have been limited to only one UAS element. By combining disparate UAS elements, we obtain surprisingly high levels of gene expression, as high as the strongest promoters previously described in *Y. lipolytica* (Blazek et al. 2011) in a much shorter total promoter size (1,850 bp vs. up to 3,700 bp). We hypothesize that the distinct UAS elements localize different transcription activating factors with unexpected cooperative effects and thus more efficiently enhance transcription. Prior evidence has demonstrated that the combination of GCR1 and RAP1 regulatory sequences constitute one of the strongest activating sequences known in *S. cerevisiae* (Drazinic et al. 1996). The UAS1B sequence was initially identified through a deletion analysis of the XPR2 promoter and was shown to be necessary for XPR2 promoter function (Blanchin-roland et al. 1994). The UAS1B sequence contains a TUF/RAP1 transcription factor

binding site that by itself rescues native promoter activity when inserted to replace a UAS1B deletion (Blanchin-roland et al. 1994). Our transcription factor deletion analysis of the consensus GCR1 binding site found in the UAS_{TEF} sequence confirmed its importance towards transcriptional activation. Hence, the unison of GCR1 and RAP1 transcription factor binding sites within the same promoter could drastically increase transcriptional capacity, and it is likely that the localization of these regulatory sequences serves as the mechanism behind the synergy seen when combining the disparate UAS_{TEF} and UAS1B₈ elements to form hybrid promoters. These types of synergies will form the basis of developing predictive models for designing de novo promoters.

UAS_{TEF#2}-based hybrid promoters retained the constitutive expression properties found in the native TEF promoter and exhibited consistent expression levels regardless of media formulation or cell phase. As the UAS_{TEF#2}(12)-TEF(136) promoter retained full activity much longer than other library members, increasing UAS_{TEF#2} copy number may serve to delay the onset of reduced promoter activity. In contrast, hybrid promoters containing only UAS1B elements (UAS1B₁₆-Leum and UAS1B₃₂-Leum) displayed a cell-phase-dependent time course expression pattern and remained only marginally active until later cell phases. The addition of a TEF_{UAS} element in the form of a TEF core promoter (UAS1B₈-TEF and UAS1B₁₆-TEF) shortened this lag period and facilitated quicker promoter activation. This phenomena was seen more prominently with the UAS_{TEF(2)}-UAS1B₈-TEF promoter. Thus, we

Fig. 7 Effect of media formulation on hybrid promoter expression. **a** Hybrid constructs were tested in medium containing glucose, sucrose, glycerol, or oleic acid as the sole carbon source. UAS_{TEF#2}-TEF(136) promoters did not significantly vary with carbon source. **b** UAS1B promoters were further activated when grown on sucrose or oleic acid and had lower levels when grown on glycerol. *Error bars* represent the standard deviation of measurements between biological triplicates



have shown that both hybrid promoter potency and regulation can be manipulated through the choice of UAS element and core element.

Sucrose-activated UAS1B hybrid promoters yielded surprisingly high expression values compared to glucose, presenting it as an alternative carbon source for protein expression. However, maximum cell density was significantly

decreased in sucrose-grown cultures. As sucrose is a disaccharide comprised of glucose and fructose, fructose-grown cultures were also analyzed for protein expression capability but generated less expression than glucose-grown cultures. Thus, further research is necessary to determine the mechanism for such high heterologous protein expression in sucrose-grown cultures.

Collectively, these results illustrate a detailed example of hybrid promoter library construction to increase promoter strength in an organism. The basic architecture of hybrid promoters informs the need for both upstream activation sequences and core promoter regions. Dissection analyses of native promoters can mediate the efficient isolation of novel UAS elements. Tandem UAS elements help bypass the enhancer limited nature of promoters by serving as transcriptional amplifiers, and fusion of UAS repeats to native promoters elevates their basal strength, and therefore cell-wide transcriptional capacity. We have demonstrated that this amplification is generic for all promoters (regardless of length) and thus not restricted to minimal promoters. Nevertheless, minimal promoters can be isolated through a general truncation and UAS replacement analysis. Minimal promoters increase the ultimate dynamic range of the hybrid promoter approach and allow for fine-tuned gene expression starting at a lower level. Exploitation of both native and minimal promoters under the control of tandem UAS elements permits an otherwise unattainable range of gene expression. Moreover, combining unrelated UAS sequences offers tantalizing potential for ever higher levels of gene expression and controllable regulation. In conclusion, the generic approach for hybrid promoter engineering advanced here is an important synthetic biology method enabling the construction of high-level and fine-tuned promoters.

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