FEBS Letters xxx (2012) xxx–xxx

journal homepage: www.FEBSLetters.org

Review

Construction of synthetic regulatory networks in yeast

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ARTICLE INFO

Article history:
Received 31 December 2011
Revised 25 January 2012
Accepted 26 January 2012
Available online xxxx

Edited by Thomas Reiss and Wilhelm Just

Keywords:
Yeast
Synthetic biology
Regulatory network
Saccharomyces cerevisiae
Regulation
Biological part

ABSTRACT

Yeast species such as *Saccharomyces cerevisiae* have been exploited by humans for millennia and so it is therefore unsurprising that they are attractive cells to re-engineer for industrial use. Despite many beneficial traits yeast has for synthetic biology, it currently lags behind *Escherichia coli* in the number of synthetic networks that have been described. While the eukaryotic nature of yeast means that its regulation is not as simple to predict as it is for *E. coli*, once initial considerations have been made yeast is pleasingly tractable. In this review we provide a loose guide for constructing and implementing synthetic regulatory networks in *S. cerevisiae* using examples from previous research to highlight available resources, specific considerations and potential future advances.

1. Introduction

In 2011, a team of synthetic biologists from the Johns Hopkins University School of Medicine announced the technological feat of engineering a *Saccharomyces cerevisiae* yeast strain with chromosome arms assembled from chemically-synthesised DNA [1]. This achievement represents the first major milestone in a global project to engineer the first synthetic eukaryote; a human-made yeast strain that will be known as SC2.0. This project potentially marks a new phase in our relationship with yeast, a microbe that has already been exploited by humankind for several millennia [2]. While this work will undoubtedly provide new avenues for improving yeast for human use, it is worth noting that the strains we work with in baking, brewing and in the laboratory are already well-suited to industrial and research applications. They are simple and quick-to-culture non-toxic cells, not only useful for a variety of different applications but also sufficiently robust that they can be freeze-dried and sold in sachets. The foremost strain used in research and industry, *S. cerevisiae*, has many inherent properties desirable for synthetic biology (Table 1) and stands in an enviable position for use in a future bio-based economy as its worldwide use in food and drinks provides it with an established global infrastructure and skill-base for its industrial use.

Synthetic biology is the application of engineering principles to the process of constructing and implementing human-designed biological systems. The diverse goals, themes and terminology of this subject have been extensively reviewed elsewhere [3,4]. Synthetic biologists essentially aim to predictably produce a wide variety of novel devices, networks and pathways through rational recombination of modular DNA-encoded biological parts. The devices and regulatory networks constructed offer a variety of applications to science, industry and healthcare when implemented and interfaced with living cells [5,6]. Since 2000, *S. cerevisiae* yeast (the subject of this review unless otherwise stated) has been used as the chassis cell for around a dozen examples of regulatory network engineering projects in synthetic biology. This puts it behind both *Escherichia coli* cells and mammalian cell culture lines in terms of numbers of published synthetic gene networks, despite its enviable properties for rational engineering and science's comprehensive and well-catalogued understanding of its biology [7]. Given that yeast re-engineering for biosynthesis by researchers and biotech companies is already underway, combined with the anticipated need to introduce programmed regulation into future metabolic engineering projects [8], developing new synthetic regulatory networks for yeast is a key goal for synthetic biology. Such networks will aid in improved production of fuels, pharmaceuticals and high-value chemicals [9,10], while also offering other applications such as biosensors and biological computation. To construct and implement synthetic regulatory networks in yeast, many of the same approaches used elsewhere in synthetic biology apply; how-

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doi:10.1016/j.febslet.2012.01.053

Table 1
Properties of *S. cerevisiae* yeast relevant to synthetic biology. A summary of general properties of yeast that are beneficial (+) and potentially problematic (–) for synthetic biology.

Property		Relevance to synthetic biology
Proficient DNA recombination	+	Effective and targeted integration into host chromosomes
	+	Proven assembly of very large DNA fragments
	–	Repetitive sequences can result in unstable constructs due to enhanced recombination rates
Many selectable markers	+	Multiple constructs can be introduced into one cell
	+	Positive and negative selectable markers available
	+	Routine use of auxotrophy for selection without antibiotics
Compartmentalisation	+	Enzymes and pathways can be targeted to organelles where toxic products or substrates can be concentrated
	–	Targeting of mRNA or proteins to compartments can be difficult
Nucleus	+	Genetic material is insulated from the rest of the cell
	–	DNA targeting proteins need effective nuclear localisation tags
	–	Modifications to mRNA sequence can affect export to ribosomes
Chromatin	+	Co-ordinated regulation of large constructs may be possible
	–	Local chromatin at integration locus affects gene expression
Genome topology	+	Yeast chromosomes naturally have rational spacing of genes, with few overlapping open reading frames
	+	<5% of genes have introns, with almost no alternative splicing
	–	No operons: every protein coding sequence needs its own promoter to be efficiently expressed
Lack of native RNAi system	+	Less unexpected regulation due to natural regulation systems
Haploid or diploid cells	+	Haploid cells offer single-gene, single-phenotype relationship
	+	Mating cells can be used in gene network construction
Suitable for industrial use	+	Growth to high density in aerobic or anaerobic conditions
	+	Can utilise a wide range of carbon sources and substrates
	+	Not susceptible to contamination by bacteriophages
Extensive existing research	+	Well-characterised genome, proteome and metabalome
	+	Bioinformatics databases are well-organised and in-depth
	+	Many engineered strain libraries available, e.g. gene knockouts

ever, some specific considerations for parts, construction and optimisation are necessary. In this review we will describe the considerations needed when constructing regulatory networks in yeast, following the process from design and simulation to construction, testing and implementation.

2. Design and simulation

As with any engineering project, the first stage of a synthetic biology project is the design; led by the problem to be solved and specifications that need to be met. Regulatory networks constructed by synthetic biology approaches have been previously used to either investigate natural biological phenomena or to endow cells with novel functionalities. In yeast, this has led to a variety of network designs implemented at the transcriptional and post-transcriptional levels in single cells and in consortia. Examples of both types of networks are highlighted in Fig. 1, along with the parts used to generate these and the methods employed to tune them.

For predictable design it is desirable to separate the network from the chassis cell's own intricate regulatory networks, whose behaviours are difficult to anticipate [4,11]. Independence from existing networks is often called *orthogonality* and is most readily achieved by using non-native regulatory parts, such as those from bacterial, viral or phage species. The first synthetic network constructed in yeast made use of an engineered version of the bacterial transposon Tet Repressor (TetR) in order to investigate how autoregulatory feedback in gene networks can lead to cell differentiation [12]. This network successfully exhibited bistability and its topology inspired a subsequent design that introduced synthetic memory into yeast cells [13] (Fig. 1A). This was later modified to endow cells with memory of DNA damage [14].

As well as offering orthogonality, TetR-based regulation also offers external inducibility through the addition of tetracycline or the related molecules doxycycline and anhydrotetracycline (ATc). For regulatory networks, inducible control of expression through external stimuli is valuable and often essential. In networks designed to give new functionality, external stimuli can be used to trigger programmed downstream responses, such as the sedimentation of cells when flocculation is directed by tunable timer networks [15] (Fig. 1B). In networks designed to probe natural phenomena, such as those used to explore the properties of gene expression noise [16–19] (Fig. 1D), inducible control offers a rapid method for tuning expression and thus rapidly exploring network space. When synthetic biology is used to investigate more complex networks, such as mitogen-activated protein (MAP) kinase signalling cascades, introducing inducible control at every node in the network would be a major undertaking. Instead, in such cases it is more important to design the synthetic gene network and even the proteins themselves to be modular so that straightforward interchanging of parts and protein domains can be used to quickly explore possible network space [20] (Fig. 1F). The use of modular part libraries at crucial positions in a network is particularly useful for rapidly generating diverse functions and outputs [15].

While these three main design considerations – orthogonality, inducibility and modularity – are common to all synthetic networks, other specifications are determined by intended use. For example, pattern-forming and quorum-sensing networks require use of multicellular systems, with cell-to-cell signalling used to achieve communicating networks in separate cells [21] (Fig. 1C). Applications in fuel and product biosynthesis require efficiency of gene expression and robust performance regardless of the environment and cell cycle phase. This therefore calls for finely-tuned constitutive expression and balancing of metabolic flux as the main specifications, along with the use of heterologous enzymes, free from native regulation [22]. In contrast, the previously described in vivo reverse-engineering and model assessment (IRMA) network (Fig. 1D) constructed as a benchmarking tool for systems biology, was specifically designed to show non-linear behaviour in order to provide a challenge for the many modelling approaches that are used to predict and simulate regulatory networks [23]. Theoretical simulations have even shown that IRMA has programmable network plasticity and can be rewired to give new networks capable of oscillations and bistability [24].

Designs in synthetic biology are largely still limited by the parts available and how easily they can be constructed and characterised, but this will undoubtedly change as the subject advances. Greater complexity in future network designs will bring a require-

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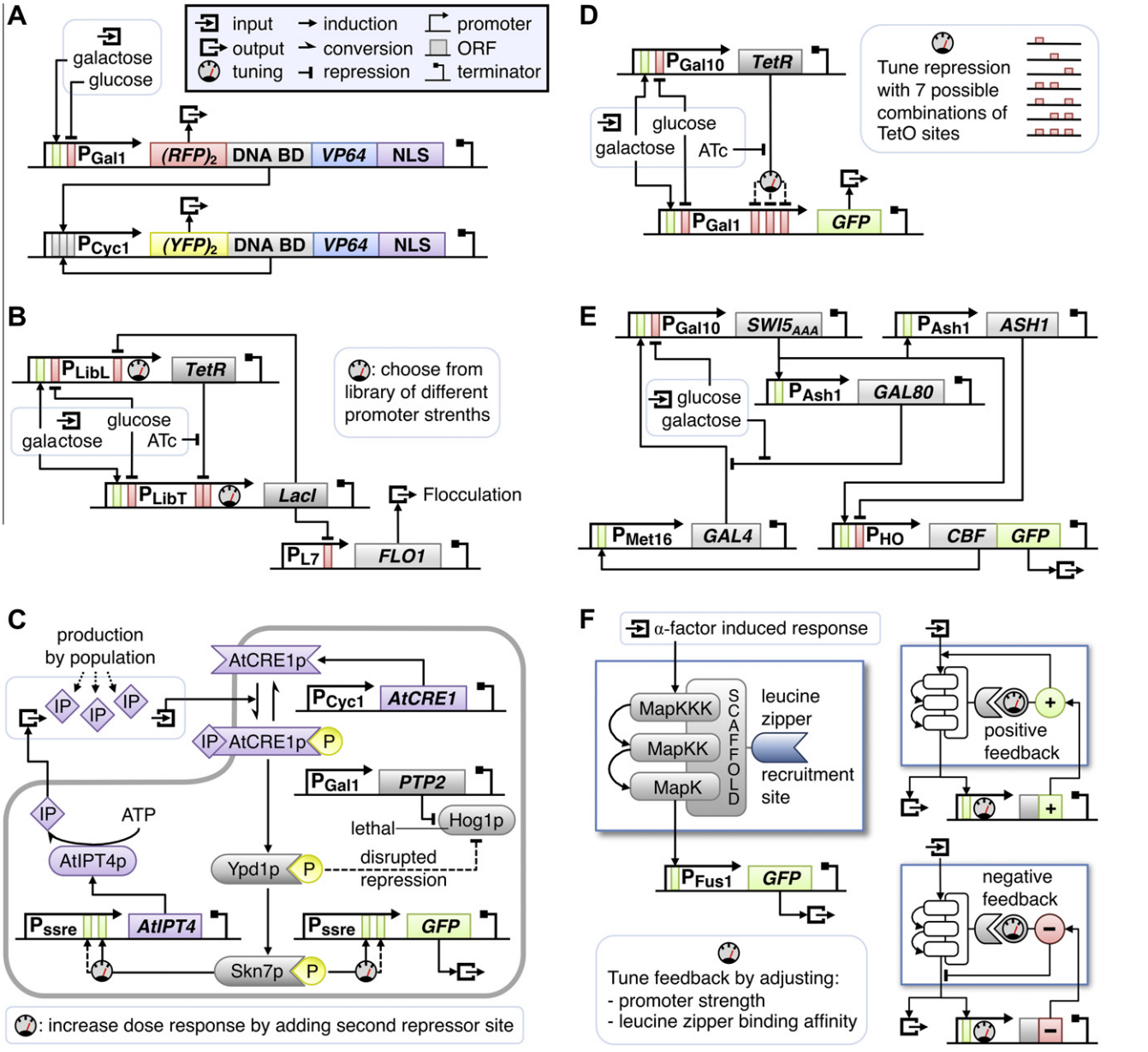

Fig. 1. Examples of yeast synthetic regulatory networks. Network diagrams show the topology and parts used to implement and tune six previously described regulatory networks in *S. cerevisiae* yeast. Unless otherwise described, all promoters, open reading frames (ORF) and resulting proteins are adapted from native *S. cerevisiae* sequences. (A) A galactose-inducible positive autofeedback loop network that functions as a heritable memory switch [13]. This network consists of two fusion proteins that include red and yellow fluorescent proteins (*RFP* and *YFP*) linked to DNA-binding domains (DNA BD), *VP64* viral activation domains and nuclear localisation signals (NLS). (B) A tunable anhydrotetracycline (ATc)-induced timer network that controls onset of the yeast flocculation phenotype via *FLO1* expression [15]. The timer network requires mutual inhibition between *E. coli* Tet Repressor (*TetR*) and lac inhibitor (*LacI*) genes, which respectively repress a library of TetR-repressible promoters (P_{LibT}) and LacI-repressible promoters (P_{LibL}). A member of the LacI-repressible promoter library (P_{L7}) is used to direct *FLO1* expression. (C) An artificial quorum-sensing network constructed by integrating an *Arabidopsis thaliana* cytokinin signalling system into native yeast signal transduction pathways resulting in production of green fluorescent protein (GFP) at high cell density. *Arabidopsis thaliana* IPT4 protein (atIPT4) is required to produce the cytokinin (IP) which is recognised by the *Arabidopsis thaliana* CRE1 protein (atCRE1) which plugs into native YPD1 signalling. A hybrid promoter containing SKN7 binding sites (P_{SSRE}) is used to regulate expression. Phosphorylated proteins in the pathway are highlighted (P) [21]. (D) A tunable ATc-induced transcriptional cascade network used to investigate the effects of regulation on gene expression noise [19]. Seven *GAL1* promoter variants with different combinations of TetR-binding sites were constructed and the subsequent effects of each configuration on expression were characterised. (E) The in vivo reverse-engineering and model assessment (IRMA) network; a modifiable inter-connected regulatory network designed to benchmark systems biology modelling approaches [23]. This network consists of highly-interlinked native regulators and *GFP*. A mutant SW15 protein ($SW15_{AAA}$) is used to allow the network to be independent of the cell cycle. (F) An alpha mating factor-responsive mitogen-activated protein kinase (MapK) signal transduction network re-engineered to be tunable and reconfigurable via modular protein–protein interactions [20]. By using a protein scaffold, components of the pathway including MapK kinase (MapKK) and MapKK kinase (MapKKK) can be post-translationally regulated to generate a wide range of cellular responses to the same initial input signal. All diagrams adapted from their original publications.

ment for mathematical modelling and simulation to aid in the design phase [3,25]. Whilst a variety of modelling techniques (e.g. ordinary, partial and stochastic differential equations) have played a central role throughout synthetic biology [26], they have been predominantly used to interpret and expand upon data taken from implemented networks. The real challenge for modelling in synthetic biology is to instead incorporate it upstream of construction; using it as a design and simulation tool. In this respect, the vision is for the plethora of mathematical techniques in use and in development to be incorporated into software for the design, simulation and optimisation of future gene networks. Already ambitious steps are being made to develop computer-aided design (CAD) tools and

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simulation environments for synthetic biology, and existing programs already offer features for automatic optimisation of heterologous gene codon usage, DNA assembly schemes and network topology design (equivalent to logic minimisation tools). While significant progress is being made in this area and has been reviewed elsewhere [27,28] it has yet to make an impact on yeast synthetic biology. Most tools have so far been developed for *E. coli* systems where recent work has elegantly illustrated how CAD can be used to quantitatively program gene expression [29]. The nearest CAD and in silico simulation has got to such model-driven engineering of yeast regulatory networks has been in the model-guided design and implementation of feed-forward loop and timers networks constructed from part libraries [15]. In this work different versions of networks can be simulated ahead of construction or after characterising an initial prototype network, as parts belonging to part libraries are well-characterised and vary only with respect to one or two key parameters that critically alter network behaviour (such as promoter output). Such work demonstrates that modelling and simulation can be a vital tool in the design and construction of synthetic networks; however, it is clear that the performance and necessity of modelling and simulation as a design tool is heavily-dependent on the quantity and quality of existing characterisation data for parts, as these provide the diversity and necessary parameter values required for simulations.

3. What parts to use?

The chief limiting factor for synthetic biology in any organism is the lack of well-characterised biological parts, and for engineering new regulation in yeast this is particularly true. Unlike for *E. coli*, research projects, competitions such as iGEM [4] and public programs like BioFAB (www.biofab.org) have not yet delivered an exponential increase in the number of standardised parts for yeast synthetic biology. Instead, what currently exist are dispersed sets of unstandardised parts scattered through recent literature. For synthetic regulatory networks, the parts of immediate importance are promoters and their regulators and the cell signalling proteins that transmit extracellular cues to the nucleus. However, recent work has also shown that RNA parts offer exciting opportunities for biological engineering.

3.1. Endogenous promoters

The lack of polycistronic gene modules (operons) in yeast places a major importance on promoter parts as each gene in a network or pathway requires its own promoter. Endogenous promoters have been used in previously described yeast synthetic networks as they have proven functionality and extensive characterisation data in diverse conditions are available for these thanks to countless transcriptomics experiments [7]. Yeast promoters typically consist of two parts; cis-regulatory elements, also known as upstream activation sequences (UAS) or enhancers, and the core promoter region [30]. Promoters generally are either *constitutive* (characterised by AT-rich sequences upstream of diffuse transcription initiation sites) or *regulated* (characterised by a TATA box motif and defined cis-regulatory elements) [31]. Commonly used constitutive promoters are the strong *ADH1* and *TEF1* promoters, while the *CYC1* promoter and the divergent *GAL1–GAL10* promoter, repressed in the presence of glucose and activated when galactose is provided, are often used as regulatory promoters. The exact beginning and end of the DNA sequence used for each promoter often varies, illustrating the lack of definition of parts in yeast. One study by Partow et al. has recently helped tackle this by providing extensive characterisation data for the *ADH1*, *TEF1*, *GAL1*, *GAL10*, *TPL1*, *HXT7*, *PGK1*, *PYK1* and *TDH3* (*GAPDH*) promoters in a synthetic biology context, giving a valuable library of key parts [32].

For synthetic gene networks, regulated promoters and the corresponding transcription factors that control these are crucial parts as they define network logic and provide the physical wires and nodes to link modular devices [11]. It is also useful if they are inducible via external cues and the *CUP1* and *MET25* promoters are regularly-used examples of these; tightly-regulated by copper and methionine, respectively, via endogenous pathways [33,34]. While native regulated promoters provide a simple solution to key parts they lack orthogonality by being plugged-into yeast's natural regulation networks. Induction of the commonly-used *GAL1* and *GAL10* promoters, for example, involves multiple native proteins in a mechanism with three feedback loops [35]. Two solutions to this problem, both used for construction of the IRMA network (Fig. 1E), are to mine existing data to reveal promoters with unambiguous regulation (e.g. P_{MET16}) and to host the synthetic network in cells where native regulators linking to yeast's own networks have been deleted (e.g. P_{ASH1}). This latter approach has also been shown to work for the *GAL1* promoter, where deletion of the *GAL2* gene changes its response to galactose from a switch-like response to a graded one [36].

3.2. Synthetic and heterologous promoters

S. cerevisiae has over 5700 of its own promoters that could be used as parts by synthetic biology, varying in length from around 150 bp to over 3000 bp. However, as yeast's native DNA repair machinery is proficient at recombining two or more identical DNA sequences longer than 40 bp [37], multiple re-use of the same parts in a cell is a concern, as homologous recombination threatens the physical integrity and long-term stability of synthetic designs [38,39]. To avoid this, it can be useful to instead employ non-native or recoded parts and several synthetic and heterologous options exist for yeast promoters. For constitutive expression, work on promoter libraries for metabolic engineering has provided a well-characterised 11-member *TEF1* promoter-based library generated by mutagenic PCR [40,41] and a 37-member synthetic promoter library constructed from key yeast expression elements such as the TATA box [42]. The viral *CMV* promoter also functions in yeast and has been used for strong constitutive expression in several studies [12]. For regulated expression, a common strategy is to construct hybrid promoters to impart synthetic regulation, for example placing the Skn7p binding sites from the *OCH1* promoter in equivalent positions within the *MEL1* promoter, yielding P_{SSRE} [21] (Fig. 1C). Another option is to create hybrid transcription factors by fusing interacting and transcription-activating protein domains to the DNA-binding domains of transcription factors such as Gal4p known to activate promoters like P_{GAL1}. Such hybrid transcription factors are routinely produced by the yeast two-hybrid assay which was developed to capture protein–protein interactions [43]. Variations of this [44], often utilising Gal4p or the bacterial DNA-binding protein LexA, have been designed to yield novel and inducible transcription factors, such as a 17β-estradiol inducible promoter [45] and a reversible red-light inducible transcriptional regulation system recently demonstrated to offer precise control of synthetic gene expression in yeast [46].

A further strategy for generating synthetic promoters, particularly used when orthogonal regulation is required, is to modify native yeast promoter sequences to include the binding sites of heterologous DNA-binders. This has been used extensively with the aforementioned TetR protein and also with the bacterial LacI protein to yield a combinatorial library of seven repressible *GAL1* promoters with 1, 2 or 3 TetR-binding sites [19] (Fig. 1D) and a repressible *ADH1* promoter bound by LacI [47]. Extending on this using the synthetic promoter library technique, Ellis et al. produced libraries of a further 20 TetR-regulated and 20 LacI-regulated promoters (Fig. 1B) that offer a wide range of expression outputs and

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varied efficiency of regulation upon induction [15]. Fusion of TetR and a mutated TetR to the viral VP16 domain also yields the tTA and rtTA synthetic transcription factors, respectively, which can activate yeast gene expression from modified promoters, such as a *CYC1* minimal promoter containing upstream TetR-binding sites [12]. The viral VP64 activating domain can also be used in this respect, and was fused to the LexA DNA-binding domain, a human zinc-finger protein (Gli1) and an engineered zinc-finger protein (ZifH) by Ajo-Franklin et al. to rationally engineer three positively-regulated promoters based on the *CYC1* minimal promoter with different upstream binding sites [13] (Fig. 1A). Such use of zinc-finger proteins and future use of the recently described TAL-effector (TALE) DNA-binding proteins [48], offers great promise for yielding future libraries of synthetic regulated promoters as the protein–DNA interactions of these can be rationally reprogrammed, paving a route for scalable orthogonal regulation where each promoter can be targeted by a unique artificial transcription factor.

3.3. RNA-based control elements

RNA in yeast appears to be limited in its roles compared to in other organisms. Yeast mRNAs typically have much shorter untranslated regions than equivalents in mammalian cells and alternative splicing is rarely used as a regulation strategy [49]. Recently it was shown that *S. cerevisiae* cells forfeited RNAi as a native regulation system due to evolutionary pressures, making them one of the few eukaryotes without this system [50]. While natural yeast may not make extensive use of RNA in its regulatory networks that does not stop synthetic biology from doing so. An impressive series of publications from Smolke and co-workers have shown that RNA structures such as ribozymes embedded in the untranslated regions of yeast messenger RNAs (mRNAs) can be used to modulate gene expression in response to a handful of ligands, both by modulating the efficiency of translation initiation [51] and by altering the stability and lifetime of the mRNAs [52,53]. For fine-tuning gene expression and for adding new points at which to control synthetic regulatory networks these RNA parts provide an invaluable resource. Further work by Win and Smolke also showed that scalable logic systems could also be implemented in yeast using modular RNA parts fitted together to act as a variety of logic gates [54,55]. This research demonstrates that synthetic gene networks in yeast need not be dependent on transcriptional regulation. Further research by the same group and others has since implemented related RNA parts to help optimise metabolic networks [56], to control alternative splicing in human cells [57], optimise gene expression in *E. coli* [29,58] and to create a bacterial kill-switch [58].The advantages of using RNA parts over protein regulators are that RNA requires fewer cellular resources to be produced and maintained in cells, RNA structures can be reasonably predicted by computational tools, RNA can be evolved to bind new ligands, and RNA–RNA interactions can be designed to be orthogonal by using base-pairing rules to design parts that only bind each another [59].

3.4. Cell signalling proteins

The native regulatory responses of yeast to external cues are typically processed via signal transduction cascades where relays of proteins rapidly pass post-translational modifications from the cell membrane to the transcription factors of the nucleus where gene expression is controlled [60]. Recent work has shown that cell signalling proteins can be engineered for synthetic biology and in several elegant studies in yeast one class of native pathways, the mitogen activated protein (MAP) kinase phosphorylation cascades [61], have been exploited and re-engineered to give novel regulatory networks. To develop artificial cell-to-cell communication in yeast, Chen and Weiss integrated *Arabidopsis* cytokinin signal synthesis (AtIPT4) and receptor (AtCRE1) components into yeast cells, tapping into the native SSK1 osmolarity-sensing cascade (Fig. 1C) to activate gene expression in response to cytokinin [21]. For operation of this modified cascade in yeast it was necessary to overexpress an inhibitor (PTP2) to suppress the lethal response of activating the endogenous cascade in normal growth conditions. Cell-to-cell communication in more recent work on distributed biological computation with multicellular systems made use of the native *S. cerevisiae* α mating factor and its equivalent from *Candida albicans* as orthogonal communication wires between engineered cells [62]. These two factors are bound by the native and heterologously-expressed *C. albicans* STE2 receptors respectively, activating MAP kinase pathways involved in mating and inducing engineered gene expression from the *FUS1* promoter. As with SSK1 pathway, predictable use of the mating pathway in this study required cells to have numerous interacting genes deleted, mutated or overexpressed. The same MAP kinase mating pathway was also re-engineered previously in a project to reshape signal cascade dynamics [20]. Ste5p is a key protein in MAP kinase cascades that acts naturally in cells as a scaffold to bring cascade proteins into close proximity and enable fast phosphorelay signal transduction [61]. By engineering leucine zipper heterodimerisation modules onto Ste5p, Bashor et al. were able to program recruitment to the scaffold of negative and positive regulators of the cascade. These were fused to a variety of complementary zippers of different affinities expressed at different levels (Fig. 1F). This enabled predictable engineering of a wide range of dynamic responses into the signalling pathway, including pulsing, delay and ultrasensitivity, illustrating the value of such zipper parts for engineering protein–protein dynamics in yeast [20]. As well as plugging-into native pathways, it has also been possible to completely transfer a minimal estradiol-activated MAP kinase cascade from mammalian cells to yeast without it cross-talking significantly with native pathways [63]. Whilst the synthetic network in this case was used to investigate signal processing dynamics, it offers hope that signalling pathways from a variety of organisms could be engineered into yeast to offer orthogonal regulation in response to a wide variety of inductions.

3.5. Other parts

To construct synthetic regulatory networks in yeast, several other part classes need to be considered and offer further opportunities for tuning gene expression and network behaviour. To avoid open reading frame read-through and to generate stable mRNAs appropriately exported to the cytosol for translation it is preferable that each gene ends with a terminator sequence [64]. Commonly-used terminators are native sequences found at the 3′ end of well-characterised genes such as *ADH1* and *CYC1*. They tend to be 50 bp to 250 bp long, repetitive and AT-rich [65]. Despite being obvious hotspots for recombination, these few terminators are regularly re-used in synthetic designs and no synthetic libraries of yeast terminators yet exist. Likewise, no library of Kozak sequences exists for yeast synthetic biology, despite this short part offering tuning of translation initiation efficiency. In *E. coli*, a biophysical model of mRNA structure at the ribosome binding site, the prokaryotic equivalent of a Kozak sequence, has led to an invaluable forward-design tool, the RBS Calculator [66] which generates synthetic DNA sequences customised to give specific translation initiation efficiencies. A recent related biophysical model of how different multicloning site sequences affect gene expression from three yeast promoters offers promise towards similar part-design tools for yeast [67]. A specific class of Kozak sequences, internal ribosome entry sites (IRES) in particular are an attractive part to develop for yeast synthetic biology as they can allow artificial

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operons to be constructed, allowing one promoter to coordinate expression of multiple genes [68]. Viral IRES sequences have been demonstrated to work in yeast [69] and a library of synthetic IRES sequences [70] has also been developed, although only providing low-efficiency of translation initiation.

A further answer to the lack of operons in yeast is the use of fusion proteins, where two or more proteins that require equivalent expression profiles are physically linked by short, flexible amino acid sequences. Fusion proteins are encoded by chimeric gene sequences made from multiple open reading frames and are similar to operons at the gene level but result in large multi-domain proteins. In several synthetic studies in yeast this tactic has been used to monitor network behaviour, by fusing green, yellow and red fluorescent protein reporters (GFP, YFP and RFP) directly to regulator proteins whose expression varies during operation [13,23] (e.g. Fig. 1A and E). Fusion proteins also offer a strategy to physically co-localise enzymes and regulators [20] (Fig. 1F) and can be used to target translocation of proteins to specific cellular environments, such as to the extracellular surface [71] and to vacuoles [72]. Subcellular localisation can likewise be directed by attaching short peptide tags to proteins, with nuclear import sequences (NLS) being the most relevant case for synthetic regulatory networks. Commonly-used tags in this case are the strong SV40 viral NLS and the c-Myc NLS. Mutations in their sequences lower import efficiencies and can be evaluated from sequence rules via the cNLS Mapper online tool [73]. Such tunable nuclear import offers the attractive option of being able to modulate transcription factor behaviour through spatial control. Peptide tags can also offer temporal control of proteins by directing the speed of their degradation. Protein stability in yeast is partly determined by the 'N-end-rule' of ubiquitin-mediated proteosomal degradation which has not yet been successfully exploited in yeast synthetic biology, but remains a potential tool [74]. As an orthogonal alternative to this, Grilly et al. successfully transplanted the ssrA-tag degradation system from *E. coli* into a regulatory network in yeast in order to direct specific degradation of C-terminal tagged proteins upon chemical induction [47]. Their imported system offers tunable protein lifetimes of specifically tagged proteins as variation in the tag sequence leads to different degradation efficiencies.

4. Construction considerations

Whilst the natural properties of yeast are particularly well-suited to DNA assembly (Table 1) it is still a major challenge to implement a synthetic network within yeast, despite major recent advances in synthesis and assembly of large DNA constructs [75,76]. The first consideration of construction is the physical hosting and arrangement of the final DNA construct that will be placed within the cell. This is heavily influenced by the network design, available parts and intended use. For hosting synthetic DNA extrachromosomally, yeast has a variety of different options that have been reviewed recently [9] which offer varied capacity, stability and copy-number. Both 2-micron and ARS origin shuttle plasmids are regularly used and for very large constructs yeast artificial chromosomes (YACs) have also been utilised [77]. Extrachromosomal systems can offer ease of use and high-copy numbers (which can help negate some stochastic effects in networks [78]) but require continual selection to be maintained. The attractive alternative option in yeast is to integrate synthetic DNA into the native genome, making use of yeast proficiency at homologous recombination [37,39,76]. This is routinely achieved using integrative plasmid shuttle vectors designed to insert at selectable sites on various chromosomes [79] but can also be performed using appropriately designed PCR products or chemically synthesised DNA fragments flanked by homologous sequences that can be designed to target almost any genomic site [37]. Genomic integration requires an initial selection to identify successes but after this offers long-term stability and (in haploid cells) predictable single-copy expression of each gene.

For selection of transformed cells, yeast has a wide variety of selectable markers, including both resistance cassettes such as *kanMX* (kanamycin), *natMX* (nourseothricin), *hphMX* (hygromycin B) and *patMX* (bialaphos) [80–82], and auxotrophic markers e.g. *URA3*, *TRP1*, *LEU2* and *HIS3*. The latter of these require working with strains with inactivated endogenous copies of these genes [79] but offer stable selection without the need to provide an external chemical. Some markers, such as *URA3*, *TRP1* and *LYS2*, also have the benefit of providing counter-selection in the presence of certain precursors of toxic compounds (5-fluoroorotic acid, 5-fluoroanthranilic acid and α-aminoadipate, respectively) [83–85]. Counter-selection provides useful methods to remove such markers after their use in initial selection, allowing them to then be re-used. Routinely-used marker-removal methods flank marker genes with homologous sequences or loxP sites and use native recombination or Cre-induced recombination, respectively, to remove the marker under counter-selection [86,87]. A recent and scar-less method of this, known as mutagenic inverted repeat assisted genome engineering (MIRAGE), uses an inverted repeat of the marker gene to catalyse more rapid self-excision [88].

For the assembly of large constructs at a single locus, recycling two or more selectable markers with reiterative recombination is an attractive, if serial, option. A variation of this technique has been used to gradually replace native chromosomal sequences with synthetic DNA in the SC2.0 project [1]. Recently a modified version of reiterative recombination was developed using two high specificity endonucleases and has shown great promise for combinatorial construction of multigene systems [89]. In many cases, however, it is not desirable to place entire synthetic constructs such as regulatory networks in one location. Such constructs typically contain repeated sequences (e.g. terminators) and are more prone to recombination when gathered at single sites than when distributed throughout the genome [39]. Instead, a typical strategy employed with synthetic biology is insertion of different sections of a network into separate genomic loci known to be stable and easy to select for. However, it should be noted that not all loci are equivalent. Recently, Flagfeldt et al. investigated 20 rationally-selected sites in the yeast genome by measuring heterologous genes expression from two promoters inserted at these loci. Despite rational selection, expression levels between loci were shown to vary up to eight-fold in a promoter-independent fashion [90]. This demonstrates that genomic context plays a significant role in synthetic gene expression; where the DNA is placed in the genome can affect its expression level and stability. This is particularly true of regions close to structural features of the genome such as the telomeres and centromeres, as well as for the nucleolus, which is formed from hundreds of repeats of ribosomal gene clusters on chromosome XII. Recent work uncovering the three-dimensional architecture of the yeast genome [91] offers hope for a greater understanding of which genomic loci are suitable to use, how much DNA can be inserted at a single site and how different sections of a synthetic network can be dispersed around the genome and maintain predictable expression. For regulatory networks, it will be particularly interesting to see if loci under the same transcriptional regulation are regularly brought together to physically interact in transcription factories [92,93].

Once the content, selection system and genomic context of a synthetic construct have been determined, an appropriate DNA assembly method is required. For integration at a single locus the aforementioned reiterative methods are suitable, but a plethora of other DNA assembly techniques are also available [94] and many are more applicable to the usual scheme where parts-based

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construction is performed in *E. coli* ahead of insertion into yeast. In particular, Gibson assembly, pairwise-selection assembly and Golden Gate assembly allow for the fusion of multiple DNA sequences whilst avoiding restriction sites and scar sequences [95–97]. This means that the function of genetic constructs, rather than construction considerations can determine their final DNA sequence, allowing the spacing of elements to be accurately controlled. Such methods also reduce the occurrence of repetitive sequences, helping to stabilise constructs from deleterious homologous recombination. Avoiding repetitive sequence is a constant design pressure when constructing synthetic networks in yeast, making it unattractive to re-use genes multiple times or use endogenous genes. Codon-optimisation during direct DNA synthesis offers opportunities here, as all open reading frame regions (not just those of heterologous genes) can be rationally recoded using synonymous codons so as not to be identical to one another.

5. Testing, optimisation and implementation

To ensure that a synthetic construct is behaving as predicted within the cell, in vivo characterisation is essential, usually measuring the output of a synthetic network by linking it to expression of a reporter gene. Whilst colorimetric assays using LacZ [98] and bioluminescent reporters such as luciferases [99] are available, in most cases fluorescent protein reporters, such as RFP and yeast enhanced GFP (yEGFP) are used. Expression of these proteins (controlled either via promoters linked to networks or via fusion to network proteins) can be quantified from live cells both at the population level via a fluorometer or on a more-accurate single cell basis using flow cytometry. A more direct measurement of transcriptional output of a network, used by Cantone et al. (Fig. 1E) is to quantify mRNA levels via reverse transcription qPCR [23]. While this offers increased accuracy in gene expression measurement it is an invasive technique requiring increased time and reagent costs to perform. Despite the concerns with homologous recombination in yeast, so far no studies in yeast have characterised long-term genetic stability of synthetic constructs, but related research in *E. coli* highlights the value of such data [38] and future work should consider this.

Characterisation data taken for a synthetic construct is invaluable for determining if it will function in its intended role, and how it could be optimised and improved upon. It is therefore desirable where possible to test a synthetic network under a wide range of conditions and use measured outputs to determine realistic parameter values for models capturing network behaviour. Thorough testing of prototype networks can yield accurate predictions of network behaviour in untested conditions [23] or guide construction of variants of the network with different properties [15,24]. It can also be useful to debug failed network designs. A simple but often viable solution in such cases is to replace one part with another more suited to the network, for example, changing a strong promoter for a weak one [21]. Parts libraries such as those for synthetic promoters are an extremely useful tool in this respect, as their high resolution of outputs offers a chance to finely optimise network expression levels [15,40–42] Increased use of rationally tunable parts, such as the RNA tools generated by Smolke and co-workers and peptide degradation tags should pave new routes for rational network optimisation. A final method for optimising synthetic networks, as yet not demonstrated in yeast, is to use directed evolution; using mutagenesis and in vivo selection to automatically yield circuits with desired characteristics [100].

Once the construction, testing and optimisation of a synthetic network are complete, it can be implemented in a wide range of applications. While many networks demonstrated so far have been used to probe natural systems and the fundamental rules behind phenomena such as feedback regulation and stochasticity [12,16–18,20,23,63], others can immediately be linked to natural cell phenotypes to give novel, regulated functionality to yeast. Networks designed and tested with GFP to act as timer switches have been used to drive programmable cell flocculation and sedimentation in yeast by replacing the *GFP* reporter sequence with the flocculation-inducing *FLO1* gene [15] (Fig. 1B). Similarly, memory networks responding to galactose (Fig. 1A) have been rewired at the inducible promoter to respond to native yeast DNA damage pathways, yielding whole-cell biosensors capable of recording exposure to alkylating agents [14]. Further work has also created a synthetic network in yeast that responds to the endogenous cell cycle stage of the cell, selectively arresting daughter cells during cell division upon chemical induction [101]. By enriching yeast populations with ageing mother cells, this synthetic network offers an interesting new tool to study ageing.

An area in which yeast synthetic biology is showing great promise is in product biosynthesis and the quintessential example of such metabolic engineering in yeast is the engineered production of artemisinic acid, a precursor in the synthetic production of the valuable anti-malarial drug artemisinin [102]. Many of the parts and devices created for synthetic networks in yeast can also be used in metabolic engineering in yeast. Re-engineered *GAL* regulation has been used to optimise gene expression in engineered benzylisoquinoline alkaloids production pathways [36] and RNA-based control parts have been used to non-invasively detect accumulation of metabolites in yeast [54] and to control flux through engineered ergosterol biosynthesis pathways [56]. Elsewhere a consortium of four engineered yeast cells has been generated from modular parts to each display different extracellular components of a mini-cellusome. This gives a population of yeast that can cooperate to convert cellulose to ethanol [71]. On a similar theme, another consortium of cells has also been created to produce fuel from cellulosic biomass, this time producing methyl halide compounds [72]. In this work, cellulose is degraded naturally by *Actinotalea fermentans* bacteria cultured in a mutually-dependent relationship with the engineered yeast cells which convert degraded cellulose into the desired product. Previous synthetic networks have also created interesting mutual dependencies between engineered yeast cells [103] and the exploitation of multicellular systems [21] offers promising ways to engineer greater complexity into both biosynthesis schemes and into synthetic regulatory networks. Nowhere has this been more acutely demonstrated than in a recent study by Regot et al. where a library of logic-function encoded yeast cells were mixed in various consortia to perform an impressive variety of logic computations [62].

6. Future prospects

Yeast's proven ability to assemble and maintain entire bacterial genomes [76] demonstrates that it has the tools and capacity for the construction and hosting of large synthetic networks. Future designs for regulatory networks are anticipated to require tens or hundreds of genes [11], making yeast a desirable chassis for such work. Although maintaining surplus DNA does not seem to be a problem for yeast, care will need to be taken in future designs to avoid issues that may arise with major changes to genome topology. In particular, a greater understanding of chromatin will be needed to regulate and maintain non-essential synthetic gene expression in the absence of selectable markers. New parts designed to control chromatin, such as enhancers and insulators found in other eukaryotic cells, could aid in preventing or programming epigenetic silencing of synthetic DNA [104]. Large-scale synthetic expression could also overburden native processes such as nuclear import and protein degradation or could create unfavourable redox processes that unbalance cellular metabolism.

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Therefore, for robust and predictable future designs in yeast synthetic biology it will be crucial to understand, quantify and model the capacity limits of the native cell. Further use of consortia and multicellularity in yeast network engineering could be used to circumvent these limits via division of labour [3].

Whilst the synthetic networks so far described have offered diverse applications, their topologies and parts used have greatly overlapped. To increase the number and complexity of synthetic regulatory networks, new parts, particularly those performing regulation, are essential. Ideally, biophysical models will eventually allow the forward-engineering of many parts, as exemplified by the RBS Calculator in *E. coli* [66], however, until then the best alternative is to generate and accurately characterise as many new parts for yeast as possible, ideally using standardised methods to produce shareable data. Promoter libraries have already demonstrated how this can be achieved and the benefits it provides, and RNA-based regulators also appear to offer a valuable arsenal for future work. Likewise, advances in engineering zinc finger and TAL-effector transcription factors is now paving the way towards rational design of libraries of orthogonal transcriptional regulators [48,105]. The challenge here will be to incorporate external inducibility into these designs. Use of the yeast three-hybrid assay offers one route to generating new inducible transcription factors [106] and importing heterologous signalling from other organisms is another alternative [21,63]. Ultimately, both synthetic sequences and natural sequences found in distant organisms such as archaea and plants offer potential for valuable new parts. Therefore new, parallel screening techniques to rapidly assess the performance of thousands of DNA parts in various networks are needed.

The rational rewriting of yeast chromosome arms for the ambitious SC2.0 project demonstrates the natural capacity yeast has for synthetic biology. In this project, all TAG stop codons in yeast will eventually be removed, which when combined with previous research using unnatural amino acids [107], will create a cell with an expanded genetic code capable of novel chemistry. Performing such diverse chemistry within yeast is likely to be the major application of synthetic biology in this chassis cell for some time, and so a future marriage of regulatory networks with metabolic engineering is an attractive idea [8,10]. Regulatory networks can provide spatial and temporal regulation of gene expression and coordinated control of populations in diverse conditions such as those encountered in industrial bioreactors. Regulatory networks also offer precise expression, and along with expected advances in CAD and modelling tools, should help deliver design-based engineering of a wide variety of future industrial yeast strains.

Acknowledgments

The authors would like to thank Yizhi Cai, Verena Siewers and all members of Imperial College's Centre for Synthetic Biology and Innovation for the useful correspondence and conversations that have contributed to this manuscript. Work in our lab is supported by the UK Engineering and Physical Sciences Research Council (EPSRC).

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