THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Metabolite biosensors for cell factory development

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Cover: Accelerating the development of yeast cell factories using metabolite biosensors.

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Abstract

Through synergy with natural sciences and engineering disciplines, biotechnology has become a broad, interdisciplinary, scientific field with many applications. One such application is the sustainable production of industrially relevant products using living systems such as microorganisms. Transforming microorganisms to cell factories is, however, a labour-intensive and cost-ineffective process, requiring many years of extensive research. Several fields together known as systems metabolic engineering, including synthetic biology, have greatly facilitated the process of customizing microorganisms to benefit human interests. Among several emerging tools are metabolite biosensors, which can be employed in high-throughput screening endeavours for identifying productive cells and in dynamic pathway regulation for optimizing metabolic systems. Developing and engineering metabolite biosensors to fit a certain application is, however, challenging.

This thesis focuses on different aspects of utilizing and engineering metabolite-responsive transcription factor-based biosensors for facilitating the development of *Saccharomyces cerevisiae* as a cell factory. To that end, we improved the dynamic range of a malonyl-CoA-responsive biosensor by i) evaluating different binding site locations of the bacterial transcription factor FapR within different yeast promoters and by ii) using a chimeric transcription factor based on a native repressor system from *S. cerevisiae*. Furthermore, we suggest the possibility of using the CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/Cas9 system to facilitate biosensor development by guiding binding site positioning. We also employed an acyl-CoA-responsive biosensor based on the bacterial transcription factor FadR to screen for genes boosting the fatty acyl-CoA levels, which are precursors for industrially relevant compounds such as fatty alcohols. The possibility of developing fatty acid-responsive biosensors based on other transcription factors, including the endogenous transcription factor Mga2, has also been addressed. Finally, we looked into the potential of developing an alkane-responsive biosensor based on a system from *Yarrowia lipolytica*. Overall, this thesis provides answers, discussions and potential future directions on using and engineering metabolite biosensors for cell factory development.

Keywords: *Saccharomyces cerevisiae*, synthetic biology, metabolite-responsive transcription factor-based biosensors, malonyl-CoA, fatty acyl-CoA, alkanes

List of publications

This thesis is based on the following publications and manuscript

Paper I: Tao Yu*, Yasaman Dabirian*, Quanli Liu, Verena Siewers and Jens Nielsen. (2019). Strategies and Challenges for Metabolic Rewiring. *Curr. Opin. Syst. Biol.* (`https://doi.org/10.1016/j.coisb.2019.03.004`).

Paper II: Yasaman Dabirian*, Xiaowei Li*, Yun Chen, Florian David, Jens Nielsen, and Verena Siewers.(2019). Expanding the dynamic range of a transcription factor-based biosensor in *Saccharomyces cerevisiae*. *ACS Synth. Biol.* (`https://doi.org/10.1021/acssynbio.9b00144`).

Paper III: David Bergenholm*, Yasaman Dabirian*, Raphael Ferreira*, Verena Siewers, Florian David and Jens Nielsen. (2021). Rational gRNA design based on transcription factor binding data. [Under review].

Paper IV: Yasaman Dabirian, Paulo Gonçalves Teixeira, Jens Nielsen, Verena Siewers, and Florian David. (2019). FadR-based biosensor-assisted screening for genes enhancing fatty acyl-CoA pools in *Saccharomyces cerevisiae*. *ACS Synth. Biol.* (`https://doi.org/10.1021/acssynbio.9b00118`).

Paper V: Yasaman Dabirian, Christos Skrekas, Florian David, and Verena Siewers. (2020). Does co-expression of *Yarrowia lipolytica* genes encoding Yas1p, Yas2p and Yas3p make a potential alkane-responsive biosensor in *Saccharomyces cerevisiae*? *PLOS ONE*. (`https://doi.org/10.1371/journal.pone.0239882`)

*co-first author

Contribution summary

Paper I. Wrote parts of the review and created the figures.

Paper II. Designed the research together with the co-first author, performed most of the experiments and data analysis, wrote most of the manuscript.

Paper III. Designed the research together with the co-first authors, carried out parts of the experiments (strain constructions and fluorescence analysis) and wrote parts of the manuscript.

Paper IV. Designed parts of the research, performed the experiments related to the candidate genes (strain constructions, measurements and data analysis), wrote most of the manuscript.

Paper V. Designed parts of the research, performed the experiments and data analysis, wrote the manuscript.

Preface

This dissertation serves as partial fulfillment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering at Chalmers University of Technology. The PhD research was carried out between September 2016 and March 2021 at the division of Systems and Synthetic Biology under the supervision of Verena Siewers. The project was co-supervised by Florian David, examined by Jens Nielsen and funded by the Novo Nordisk Foundation (grant no. NNF10CC1016517).

Yasaman Dabirian
March 2021

Contents

List of figures and tables

Acknowledgements

It is easy to think that PhD studies are all about science, especially for a novice PhD student who may get overwhelmed in the process of trying to understand and digest the vast literature with the ambition to keep up with the fast advancements. What may not always be so clear during such periods is that anything achieved comes from having great people around, making PhD studies so much more than just science. I am grateful to have been part of an incredible group and for all the opportunities and support I have been given to reach this point.

Thank you Verena and Florian for your support throughout my studies and for always creating a friendly and supportive environment. It has been a pleasure to know you as colleagues and as friends. Jens, thank you for taking me into your group, it has been an invaluable experience. It has always been inspiring talking with you and learning from your effective leadership.

The projects I have been working on would not have been possible without the collaboration with excellent people and their guidance. Paulo, thank you for joining the fatty acid screening project, your expertise made it easier to finalize this work. Xiaowei, thank you for all our fruitful discussions and for the work on the sensor optimization project. Tao, writing the review with you was a very nice experience, you have taught me a lot. David, thanks for our collaboration and for answering my many questions about transcription factors. Max, working with you has been great! A lot of fun, a lot of learning and most importantly, a lot of enthusiasm. Christos, thank you for your work on the almost impossible alkane project. Kate, I am thankful for your genuine care and interest in my progress and for helping me to advance in all possible ways. Sharing the office with you was absolutely amazing! I am also grateful to Quanli, Olena, Rui, Yi, Jiufu, Xin and Boyang for all the discussions and help.

To the former and current members of the Sysbio family, thank you all for creating an amazing atmosphere to be in. You are all great, hard-working, people who have shown that despite the demanding workload, especially at peaks of stress and inconvenient hours, there is always room for kindness and time to help. I also enjoyed many discussions, activities and afterwork events with so many of you. Thank you for making so many moments memorable!

I am grateful to many dear friends, particularly my sister Nina, who have made life's journeys more exciting, less scary and, above all, made sure I do not take myself too seriously.

My deepest gratitude goes to my mom and dad for the endless, unconditional, love and support. Thank you for laying the foundation for a sweet life through generosity, kindness and all the incredible and adventurous times. I am also forever grateful to my dad for being the *greatest* teacher I have ever had. I cherish all our discussions. Thanks to my brother for *always* making me laugh wholeheartedly and for always being there and being protective of me since my very first steps. Finally, many thanks to my *partner*, *friend* and *beloved husband* for the joy, enthusiasm and love that gives my life an extraordinary dimension.

-Yassi

To my *mom* and *dad*

Our best thoughts come from others
-Ralph Waldo Emerson

PhD thesis outline and focus

As we are witnessing incredible advancements in science and technology today, we are likewise witnessing unprecedented environmental challenges, potentially threatening our very existence. The consequences and effects of exploiting our planet's resources could not have been more clear; global warming, polluted oceans, loss of habitats due to deforestations and extreme wheathers among many other critical issues. Many of these issues are caused by our extensive use of fossil fuels. Producing our everyday chemicals and materials from renewable non-food biomass can help reduce our dependency on petroleum-driven production. Using microorganisms to produce chemicals and other important products such as primary- and secondary metabolites, biopharmaceuticals, bioplastics and biofuels through bio-based manufacturing has shown to be of great importance [1].

Microbial biotechnology, a subfield within biotechnology, has over the last few decades achieved tremendous advancements in engineering microorganisms to cell factories. The development of cell factories is, however, challenging, expensive and labour-intensive. There is, therefore, immense research being carried out to develop tools, methods and computational models to accelerate the process of developing cell factories and make it as cost-efficient as possible. An emerging tool that has shown great potential are metabolite biosensors, which can be used in high-throughput screening to efficiently and quickly identify productive cells from unproductive ones, or which can also be used in dynamic pathway regulation to optimize the metabolism of engineered cells.

The main focus of this thesis has been in synthetic biology, specifically on developing transcription factor-based biosensors for accelerating the development of *Saccharomyces cerevisiae* as a cell factory. The thesis summary has been divided into the four following parts:

Part one: The first part gives a brief and general background on biotechnology, which is followed by a more thorough introduction of microbial biotechnology, specifically systems metabolic engineering, which will partly be based on our review paper "*Strategies and challenges for metabolic rewiring*" (**Paper I**). In this section, strategies and challenges for metabolic engineering are discussed to put the aim of this thesis in context. Chapter 1 of this thesis ends by discussing the challenges of lacking sufficient high-throughput screening tools, for example genetically encoded biosensors, that can match the pace the field has

achieved in designing and building strains. Chapter 2 focuses on different types of genetically encoded biosensors, including their potential applications for metabolic engineering. Finally, this chapter ends by specifically addressing transcription factor-based biosensors. At this stage of the thesis, sufficient background information should have been given to justify the aim and work of this thesis (Figure 1).

Part two: In part two, more details regarding transcription factor-based biosensors will be provided, specifically discussions regarding custom-made transcription factor-based biosensors in order to put the work of our study "*Expanding the dynamic range of a transcription factor-based biosensor in Saccharomyces cerevisiae*" (**Paper II**) in additional context. Following this, we discuss our study in **Paper III** "*Rational gRNA design based on transcription factor binding data*". Although this paper does not have a focus on biosensors, but instead on the expansion of how gene editing with the CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/Cas9 system may be used, we suggest that some strategies here could be used to facilitate biosensor development by guiding binding site positioning.

Part three: In part three, discussions regarding biosensor applications will be covered and demonstrated with both unpublished and published work, including "*FadR-based biosensor-assisted screening for genes enhancing fatty acyl-CoA pools in Saccharomyces cerevisiae*" (**Paper IV**) and "*Does co-expression of Yarrowia lipolytica genes encoding Yas1p, Yas2p and Yas3p make a potential alkane-responsive biosensor in Saccharomyces cerevisiae?*" (**Paper IV**).

Part four: Finally, part four of this thesis will end by first concluding the work of this four year PhD research followed by an outlook. Overall, the aim is to provide answers, discussions and potential future directions on using metabolite biosensors for cell factory development.

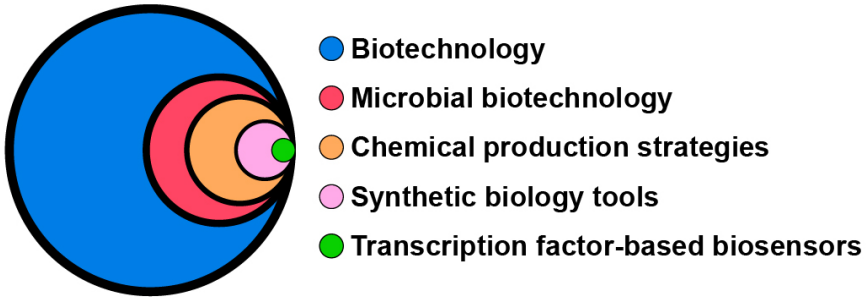

Figure 1 Main focus of this thesis. Developing and engineering metabolite biosensors, specifically transcription factor-based biosensors, in *S. cerevisiae* has been the main focus of this thesis, which is put into context as well as in a broader relation to the biotechnology field.

PART ONE

Why genetically encoded biosensors matter

1 | Background

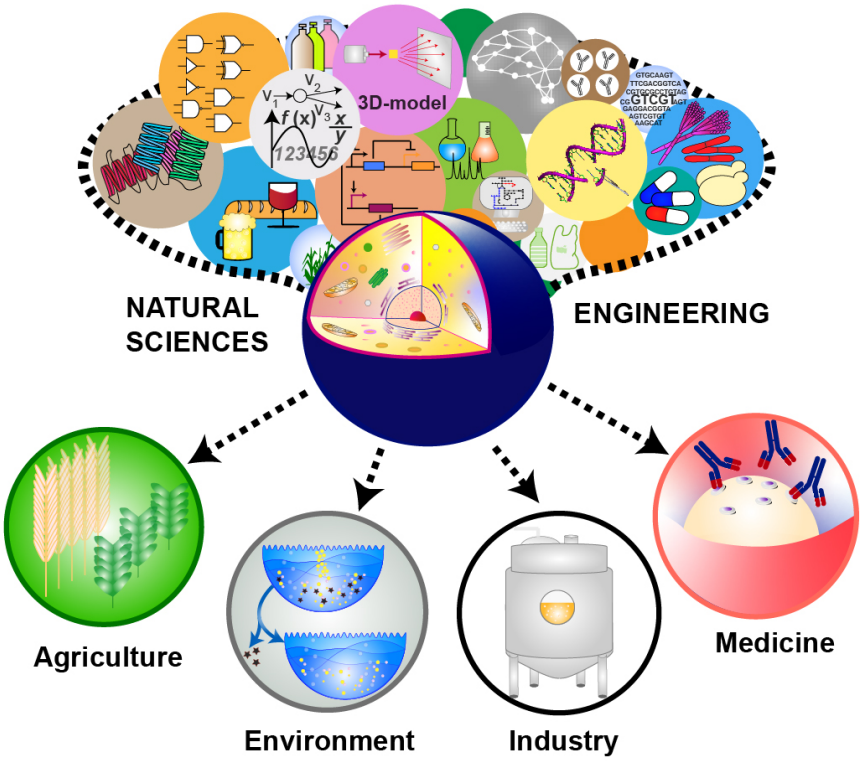

Figure 1.1 Overview of biotechnology. Biotechnology is an interdisciplinary scientific field based on natural sciences and engineering disciplines, and its core lies in using living systems. All applications commonly fall within four main fields, including agricultural, environmental, industrial and medical research.

The core of biotechnology lies in utilizing living systems to develop technologies for producing goods or services that are of human interest and benefit. Biotechnology is an interdisciplinary scientific field based on natural sciences and engineering disciplines such as biochemistry, mathematics, electric engineering, analytical chemistry and computer sciences (Figure 1.1). Although the term biotechnology was coined only a century ago by Károly Ereky [2], the field has an ancestry stretching back thousands of years when it was predominantly used in brewing, animal and plant domestication and the improvement of these through selective

breeding [3]. Since then, particularly in the 20th century, the field has witnessed great expansion, exemplified by the acetone-butanol-ethanol fermentation process during World War I and the production of penicillin during World War II, and has since the early 1970s been brought to the forefront of science with the advent of recombinant DNA technology [1, 4, 5].

Recombinant DNA technology, also known as genetic engineering, is the creation of new, biologically functional, DNA molecules by bringing together genetic material from different sources [6]. Biological systems are mainly engineered by modifying certain genotypes to obtain different phenotypes, for example, a different function or morphology of an organism. In contrast to traditional breeding methods, genetic engineering allows directed modifications of an organism's genome, then referred to as genetically modified organisms, and offers more precise and faster modifications while preventing undesirable changes to be introduced. Among the first organisms to be genetically modified was the well-characterized bacterium *Escherichia coli*, which shortly after was engineered to produce human insulin [2]. The concept of genetically modifying living systems to obtain desirable features and properties was further translated into engineering crops such that these have enhanced nutritional value, longer shelf life and increased resistance to environmental perturbations, for example drought, frost or insect pests [7,8]. Furthermore, genetic engineering has also been instrumental in environmental research, for example in bioremedition where microorganisms are used in contaminated areas to degrade pollutants [9]. Biotechnology has also advanced medical research from producing pharmaceuticals using living systems to potentially enable treatment of genetic disorders by, for example, combining stem cell technologies with recombinant DNA technologies [10]. The mentioned examples only illustrate a few applications, and biotechnology-based applications are steadily increasing in agricultural, environmental, industrial and medical research (Figure 1.1) [9].

1.1 Chemical production strategies in modern day biotech

In the decades following the introduction of genetic engineering, microorganisms have been engineered to produce several different compounds ranging from commodity chemicals, for example, biofuels, to fine chemicals and pharmaceuticals, for example, terpenoids, insulin and vaccines [11]. Developing microorganisms, also referred to as cell factories, for such purposes is the main objective of metabolic engineering, a subfield within industrial biotech.

There are several advantages of using microorganisms in industry [1]. For example, microbial cell factories have the potential to reduce our dependency on petroleum-derived or plant-extracted products and enable, for example, the production of pharmaceuticals that are otherwise too complex to be produced using traditional chemical synthesis methods [12, 13]. In fact, the importance of bio-based materials is becoming increasingly evident as, for example, can be witnessed from new regulatory rules in Europe banning single-use plastics, such as cutlery, plates and straws [14]. To advance further in this mission, there are, fortunately, already several well-characterized and established biotechnological workhorses, including

the well-known microbes *E. coli* and *S. cerevisiae*, which are unicellular microorganisms commonly representing prokaryotic and eukaryotic cells, respectively [15].

Developing cell factories, however, is often labour-intensive and cost-ineffective, mainly due to microorganisms being rewired to survive and proliferate rather than optimized for producing a compound of human interest at a commercial level. The inherently complex metabolic networks that make up a living system, and our limited understanding of it [16,17], has made biological engineering one of the most challenging parts of developing microbial factories [15]. Therefore, understanding metabolism is a prerequisite for engineering purposes, and the deeper the understanding the more predictively can biological systems be engineered.

1.1.1 Understanding metabolism for engineering purposes

A biological system is a dynamic living system capable of functioning in a certain environment while responding and adapting to changes in the surroundings. Naturally, such a system must be based on complex, tightly regulated and highly interconnected networks in order to maintain a balance between stability, flexibility and evolvability [18, 19]. The complexity of metabolism can be understood from the bow tie structure, a biological architecture found in all living systems, where a wide range of carbon and energy sources are converted into 12 precursor metabolites for producing all cellular building blocks, for example amino acids, nucleotides, fatty acids and sugars (Figure 1.2A) [20]. In order to maintain a dynamic metabolism, the activity of the metabolic pathways must be coordinated by sensitive means of communication in which allosteric enzymes are predominant. For example, the metabolism must be regulated by controlling the amounts of enzymes, their catalytic activities and the accessibility of substrates. One key point for regulating the protein abundance, is gene regulation.

Gene regulation occurs in all living systems and is necessary for producing the right proteins in the right amount and at the right time, making the living machinery possible to function, adapt and evolve. In higher eukaryotes, or multicellular organisms, gene regulation allows a single cell to be differentiated into different cell types despite all cells having the same genome, ultimately resulting in muscle cells, fat cells, blood cells and so on. Despite the similarities in the fundamental principles of these regulatory systems in prokaryotes and eukaryotes (Figure 1.2B), there is a significant difference in their complexity. For instance, while transcription and translation occur in the same compartment in prokaryotes, these processes are separated in eukaryotes due to their compartmentalized, membrane-enclosed, organelles. Additionally, in terms of genome complexity, eukaryotes have higher DNA content, several linear chromosomes, compared to prokaryotes, having often one circular chromosome (Figure 1.3A) [21]. Furthermore, unlike the "naked" DNA structure in prokaryotes, eukaryotic DNA is wrapped around nucleosomes consisting of proteins called histones, and this packaging creates a structure referred to as the chromatin (Figure 1.3B). The chromatin structure is the most important structural difference between prokaryotic and eukaryotic DNA, especially

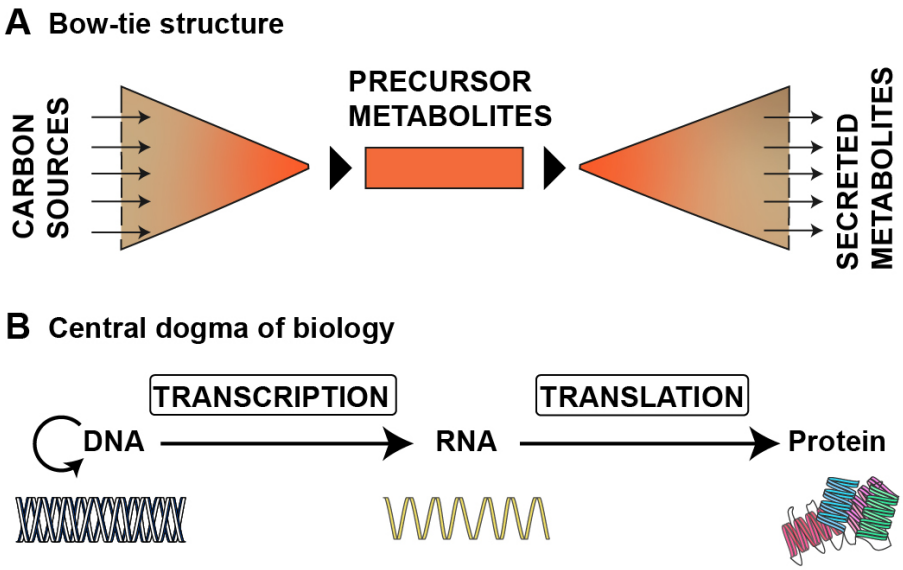

Figure 1.2 Fundamental biological similarities in living systems. (A) All living systems have at least one fundamental feature in common, the bow tie structure, where a wide range of carbon sources, such as glucose, xylose, glycerol and natural gases, are converted, through 12 metabolite precursors, into all biological building blocks, such as fatty acids, sugars, and amino acids. (B) The overall fundamental principles of the central dogma of biology is also shared between all living systems.

as it results in different transcriptional ground states. For example, the default state of transcription in prokaryotes is generally "on" whereas in eukaryotes it is considered to be "off" [22]. Often, extensive modification of the chromatin structure is required for gene expression to occur, adding an additional level of regulatory complexity in eukaryotic systems [22, 23].

For transcription initiation to occur, several key players have to be involved. In eukaryotes, this includes chromatin remodeling- and modifying complexes, RNA polymerase II, general- and specific transcription factors and their associated coregulators. In general, there must be an interplay between several key players, and the key interface between genetic regulatory information and RNA polymerase transcriptional machinery is believed to be gene-specific transcription factors [24]. Gene-specific transcription factors play an important role in mediating transcription by, for example, interacting with other regulatory proteins to localize RNA polymerase to the promoter of the gene to be transcribed. Due to their monitoring and regulatory capability, transcription factors play an important role in physiological adaptation by controlling gene expression at the transcription level. Transcription factors are themselves regulated by several mechanisms, including protein-protein interactions, protein modifications, phosphorylation or upon interacting with small effector molecules, affecting either their expression levels or their activity.

In both eukaryotes and prokaryotes, the transcriptional regulatory network is hierarchial, meaning that there are, for example, global regulators, master regulators and local regulators,

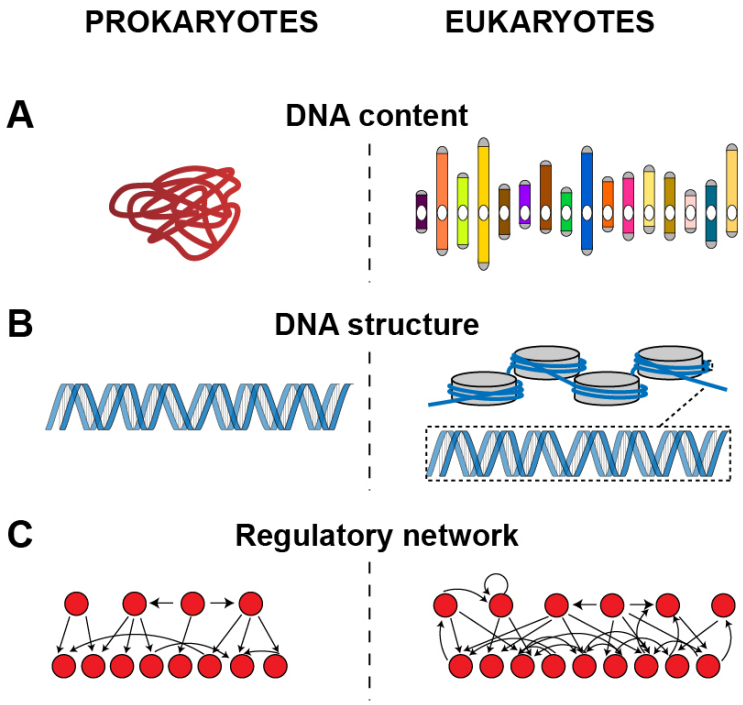

Figure 1.3 Biological differences between prokaryotes and eukaryotes. (A) Prokaryotes often have one, circular, chromosome, whereas eukaryotes have several linear ones, resulting in a more complex genome. (B) Eukaryotic DNA is wrapped around nucleosomes consisting of proteins called histones, creating a chromatin structure that does not exist in prokaryotes. (C) The regulatory network is more flat in eukaryotes due to increased cross-regulation and overlapping functions.

that have different levels of regulatory effect [25]. In prokaryotes the hierarchy is, however, more evident compared to eukaryotes where the transcriptional reguatory network is more flat, having higher degree of cross-regulation and overlapping functions (Figure 1.3C). Obviously, depending on the host strain, rewiring metabolism can have different degrees of challenges, influenced by the abovementioned factors.

In general, the better metabolism and regulatory interactions are understood, the more efficiently can strains be engineered for a desired purpose [26]. In fact, tweaking the regulatory mechanisms is an important approach to ensure that the microorganisms behave according to the production purpose. Necessary insights into cellular metabolism and physiology can be achieved through, for example, transcriptomic, proteomic and metabolomic measurements combined with integrative analysis. It is, therefore, of importance that tools, methods and computational strategies developed from different fields, such as synthetic biology and systems biology, work in synergy to elucidate the complexity of metabolism. As working on an increased systematic and global level is also necessary for efficient cell factory development, metabolic engineering has, together with other emerging fields, advanced into systems metabolic engineering [27–29].

1.1.2 Cell factory development

Systems metabolic engineering has grown to become a new area of study due to the interdependent need of systems biology, synthetic biology, metabolic engineering and evolutionary engineering to develop industrially relevant cell factories [9] (**Paper I**). Systems biology takes a holistic approach to studying biological systems, using mathematical modelling and data-driven technologies such as multi-omics analysis with the aim of eventually being able to predict cell metabolism *in silico* [20, 29]. In metabolic engineering, rational engineering is employed on a genome-scale level due to the highly interconnected nature of metabolism, making it often necessary to target several pathways before being able to re-direct metabolic fluxes towards a desirable outcome [30]. To engineer on a genome-scale level, a detailed understanding of metabolism is beneficial in order to efficiently target the right pathways. Furthermore, it is particularly important that modified and heterologous pathways are dynamically regulated such that these are well-integrated into the native metabolism. This is important in order to use cellular resources efficiently and balance metabolism such that, for example, accumulation of toxic intermediates and production of unnecessary proteins does not occur [31, 32]. As the complexity of biological systems often reveals itself when rewiring cellular metabolism for developing cell factories, methods and tools developed in systems biology can help to make metabolic rewiring more predictive [26]. However, until biological engineering becomes truly predictive, cell factory development will require several rounds of engineering and analysis until a proof-of-concept strain is developed (Figure 1.4) [15, 33, 34].

The approaches taken for engineering strains are determined by the product one aims to produce, making product selection the first step to consider in chemical production strategies (Figure 1.4). The choice of product also influences the choice of host organisms [1], as some microbes, such as *E. coli* and *S. cerevisiae*, are more suitable to use for producing fuels and chemicals whereas other microorganisms, such as *Aspergillus niger* and *Bacillus subtilis*, are more appropriate to use for producing industrial enzymes due to their efficient protein secretion machinery [15]. Although the portfolio of host strains is expanding beyond the commonly used microorganisms, the choice of a producer strain is still restricted as only a few microorganisms have i) a well-characterized system, ii) databases with information on their genome and different omics data as well as iii) tools and methods to develop these into cell factories [9, 26]. Therefore, production of a desired compound is commonly performed using well-known microorganisms, which requires that these are engineered to include the necessary pathways and optimized to achieve high titer, rate and yield while ensuring a viable production host.

The performance metrics, titer, rate and yield, are important to consider for achieving an economically feasible bioprocess. Economic feasibility can be heavily dependent on the value of the final product. For example, in industrial settings, the titer is important in order to reduce costs in downstream separation, specifically for high-value products with small quantities, whereas the yield is particularly important when the cost of feedstock in the

process is the dominant cost, for example in low-value commodity chemicals such as biofuels. Furthermore, the rate is important to reduce capital investments and operating costs [9]. These parameters need to be optimized after a proof-of-concept strain has been developed, requiring additional rounds of engineering and analysis [15, 34] (Figure 1.4).

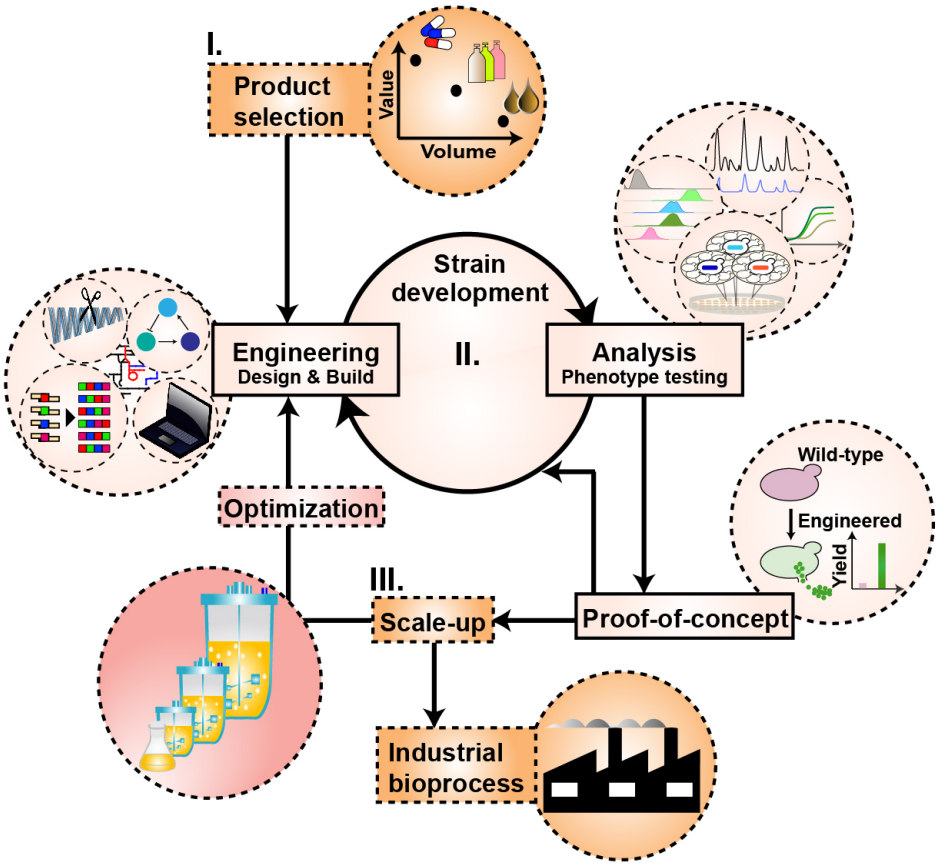

Figure 1.4 Cell factory development. Cell factory development can be divided into three sections; (I) product selection is the first step to consider as it will determine the choice of host and engineering approaches taken, (II) strain development requires several cycles of engineering and analysis in order to develop a proof-of-concept strain and (III) optimization is often needed to ensure high titer, rate and yield while scaling-up. If scale-up is economically feasible, the system can be set up for industrial applications.

There are several ways to engineer strains to meet a predefined outcome, and gene regulation is often a key parameter in engineering endeavours [35]. Traditionally, and commonly, basic DNA elements such as promoters and gene copy number are engineered as these are the foundational elements even when employing more advanced methods and tools to optimize gene- and pathway expression levels [36]. Synthetic biology has greatly advanced such engineering approaches by providing methods and tools to assemble DNA into complex pathways [9, 37, 38] and integrating these efficiently into the genome [39] such that these are optimally expressed. For example, dynamic pathway regulation has enabled carbon fluxes to

be rechanneled from redundant pathways to the one(s) of interest while ensuring a proper balance between biomass and product formation [40]. Despite these advancements, biological engineering is mainly challenging due to the uncertainty of not knowing which pathways to target or the challenge of targeting these efficiently such that expression levels are optimized [41, 42]. Using only rational engineering will often be inefficient and insufficient, and therefore high-throughput engineering approaches are becoming increasingly common.

High-throughput engineering, including random mutagenesis and large-scale library construction methods, are important for identifying key engineering targets not intuitively recognized as promising for reaching an outcome of interest [43]. Another important approach is evolutionary engineering, which follows the principle of natural selection where cells with beneficial mutations survive under selection pressure. This approach is often combined with omics analysis, predominantly genome sequencing, to identify the underlying mutations giving rise to the observed phenotypes [44, 45]. Mutations can either naturally evolve through the cultivation process, or be imposed artificially by the researcher prior to the cultivation through directed evolution methods. Directed evolution is a method generating mutations through mutagenic amplification of a desired gene using polymerase chain reaction [46]. However, the challenge here is that the production of many compounds of interest are not growth-coupled, making it necessary to develop a system that specifically couples production to improved growth. In fact, a common challenge when employing high-throughput engineering is to distinguish promising cells from millions of unproductive ones.

Commonly, strains that have been engineered to produce a certain compound are validated by analyzing their production capability using conventional analytical methods, such as chromatography or mass spectrometry. Although these methods are accurate and offer quantitative as well as qualitative measurements, the issue is that such measurements are often expensive, time-consuming and laborious [47]. Furthermore, the low-throughput nature of these methods becomes a challenge when analyzing large-scale libraries consisting of up to several millions of different variants. In a few, naturally occurring cases, quantitative analysis of non-promising strains might be avoided if the desired phenotype translates into something conspicuous, such as a chromophore or improved survival fitness under selection. There are also cases where the desired compound can be stained [48, 49], allowing for high-throughput screening using flow cytometry. The limitations here include staining efficiency and potential dye diffusion. However, since the outcome of most frequent engineering targets does not result into phenotypes that are translated or modified into something that can be easily screened for, there is a need for quantitative methods that can convert any kind of phenotype into a readable output signal [47].

The lack of high-throughput screening tools have resulted in an imbalance in the engineering and analysis cycle [33, 47] (Figure 1.4). This imbalance can, however, be mitigated in part by developing tools such as genetically-encoded biosensors, including metabolite biosensors. Metabolite biosensors, which fall within the realm of synthetic biology, have shown great potential in facilitating high-throughput screening endeavours [50].

2 | Genetically encoded biosensors

A biosensor can broadly be defined as *"any molecular device or structure that can sense a molecule of interest and output a detectable signal in response"* [51, 52] (Figure 2.1). Living systems are rich in intrinsic sensors as these play important roles in maintaining a highly dynamic, yet tightly regulated, metabolism. For example, signaling and regulatory systems responsive to environmental signals, extracellular chemicals and intracellular metabolites are an inspiring source for developing genetically encoded biosensors for metabolic engineering applications, specifically for high-throughput screening and for dynamically regulating metabolic pathways [50, 53].

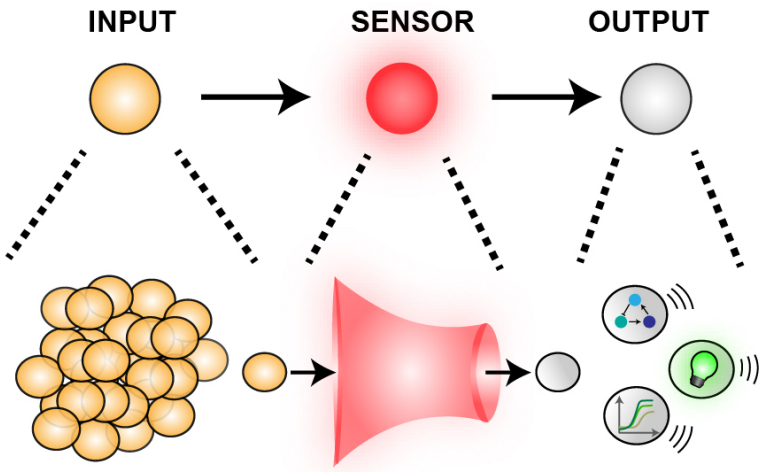

Figure 2.1 Standardizing phenotypes and regulating genotypes using biosensors. Ideally, using metabolite biosensors would enable any compound of interest to be translated into easily visible phenotypes, such as fluorescence or improved growth fitness. Furthermore, biosensors are also the basis for dynamic pathway regulation, which is desirable to have to optimize production levels of a specific compound.

2.1 Standardizing phenotypes and regulating genotypes

Using metabolite biosensors in high-throughput screening or selection would, ideally, enable a wide range of compounds to be translated into a few easily analysed phenotypes, for example, a fluorescence signal or improved growth fitness under selection pressure (Figure 2.1). This would enable phenotypes to be standardized such that more sample can be semi-quantitatively analysed in less time and using less resources than traditional methods [54]. Similarly for dynamic pathway regulation, it is desirable to regulate any specific pathway based on any compound of interest (Figure 2.1).

2.1.1 Dynamic pathway regulation

One of the first applications of metabolite biosensors for metabolic engineering purposes was in dynamic pathway regulation [55]. In this study, the concentration of acetyl phosphate was used as an intracellular indicator of excess glucose levels in *E. coli* [55]. The expression of a lycopene biosynthetic pathway was linked, through a modified version of the endogenous Ntr regulon, to elevated acetyl phosphate levels, thus increasing lycopene production. This study illustrates that expression of a heterologous pathway should not only be regulated by the concentration of pathway precursors, intermediates or end products but also by signals representing the general metabolic state of the cells and/or the growth state of the culture. In other cases, the aim is to decouple the growth phase, during which the cells produce primarily biomass, from the production phase, during which the metabolite of interest accumulates (Figure 2.2A). This is considered beneficial as implementation of heterologous pathways or modification of endogenous ones often results in static regulation, which can result in imbalanced biomass- and product formation, metabolic burden and a potential toxic state [56]. Although implementation of dynamic pathway regulation has been successful in several studies, for example improving fatty acid levels [57], 3-Hydroxypropionic acid [58] and glucaric acid [59], its application has generally been limited, which is mainly due to lack of biosensors or lack of sufficient understanding of cell metabolism to efficiently integrate pathways for dynamic regulation.

2.1.2 High-throughput screening

Biosensors are also instrumental for screening large-scale libraries [60] (Figure 2.2B), such as libraries generated using random mutagenesis approaches or overexpression libraries, which have, for example, enabled the identification of genes previously not known to improve the production of malonyl-CoA [61] and fatty acyl-CoA (**Paper IV**). Furthermore, although evolutionary engineering has been key in evolving strains with beneficial characteristics, the majority of such methods are growth-coupled, meaning that the production of a desired compound or tolerance to a certain chemical is translated into improved fitness under selection pressure. However, in cases where increased amounts of a certain compound is growth-

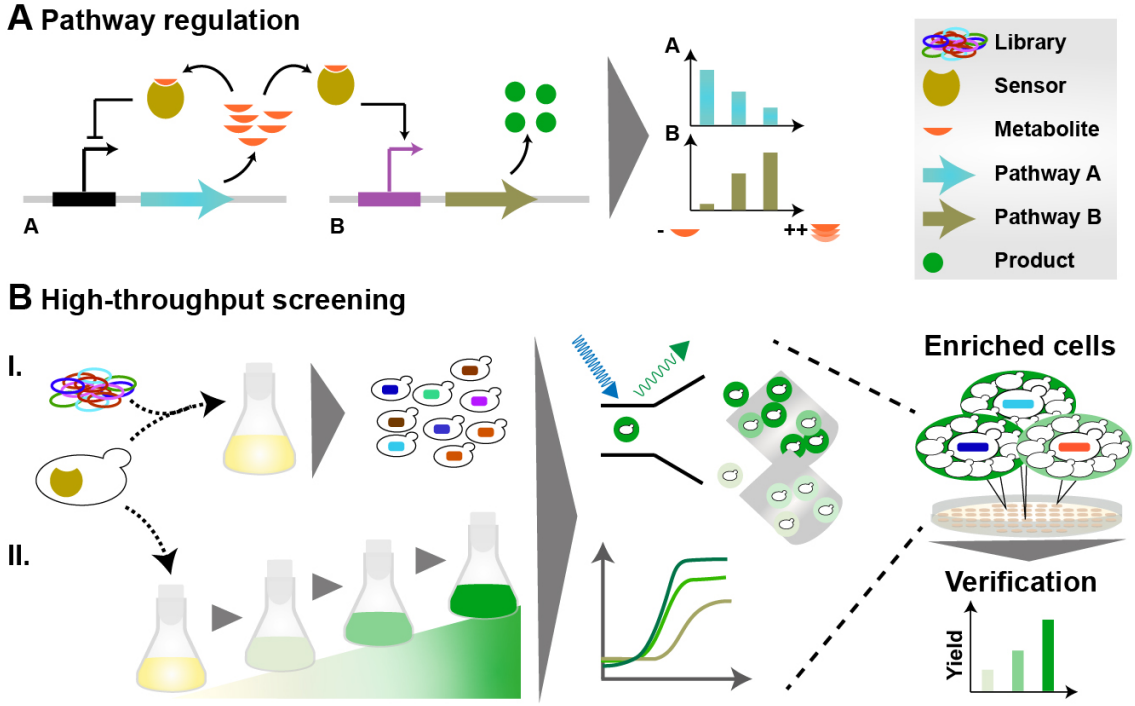

Figure 2.2 Applications of biosensors. In systems metabolic engineering, biosensors have two main applications, dynamic pathway regulation and high-throughput screening. (A) Dynamic pathway regulation enables an optimal balance between biomass and product formation. (B) Library screening and evolutionary engineering approaches combined with fluorescence-activated cell sorting or improved growth fitness under selection pressure can help identifying promising strains and, thus, reduce the number of samples that can be feasibly analysed using more accurate, traditional, methods such as mass spectrometry.

decoupled, biosensor-driven evolutionary engineering [51] has been able to couple the desired compound to increased fluorescence signal [62, 63] or to improved growth fitness under selection pressure [64].

It is, therefore, important that biosensors are well-integrated into approaches facilitating strain development as previously mentioned, for example, efficiently balancing biomass and product formation (Figure 2.2A) and finding the most promising strains in library screening and evolutionary engineering approaches (Figure 2.2B). In order for a biosensor to be useful in any of the aforementioned applications, it needs to exhibit certain features [65–67].

2.2 Features of biosensors

The performance of a biosensor is determined by several features, which are critical to consider when developing biosensors for metabolic engineering applications. Although a digital-like behaviour could be useful for dynamic pathway regulation, it is commonly not desirable to have for high-throughput screening. The aim in high-throughput screening is to distinguish high producer cells from low producer ones, and in order to distinguish between these it is important to have a graded output signal in response to increased concentrations of the desired compound. Therefore, an analog-like response with graded output signal in response to an input signal is sought for. This is often referred to as a response curve, which is used to validate the performance of a biosensor [68].

2.2.1 Biosensor specificity and response curve

An important feature of biosensors is the specificity, which is needed in order to ensure recognition of a desired compound. However, in cases where the compound of interest does not share any similarities with other compounds in the host, the specificity might not be the most determining factor for its applicability [66]. Other important features includes the dynamic- and operational, range, which can determine the shape of the response curve (Figure 2.3A). The dynamic range is commonly defined as the signal-to-noise ratio, and is influenced by the leakiness and the inducibility of a biosensor [69]. The operational range is the range of concentrations resulting in gradual changed output signal. These features are to some extent related as a low dynamic range will prevent signals from producer strains to be distinguished from any potential background noise and thereby also preventing incremental improvements of a broad range of concentrations to be distinguished (Figure 2.3B). However, a high dynamic range does not necessarily result in a broad operational range, and this is mainly determined by the sensitivity of the biosensor. The sensitivity of a biosensor is defined by the slope of the response curve, and a highly sensitive biosensor will result in a steep curve, thereby reducing the operational range. There is therefore a trade-off between these two. Furthermore, the orthogonality is also important to consider, such that the biosensor does not crosstalk or interfere with other regulatory proteins or elements in the host cell [70]. Low orthogonality prevents robust and predictive signal output, which will make biosensors useless for their purpose.

As these features are necessary to consider, naturally existing sensory molecules, or synthetically created ones, have to be engineered and custom-made if relevant features are not fulfilled. Therefore, when developing biosensors, the ease of improving these features is often considered, which also influences the type of biosensor one decides to apply for a certain application.

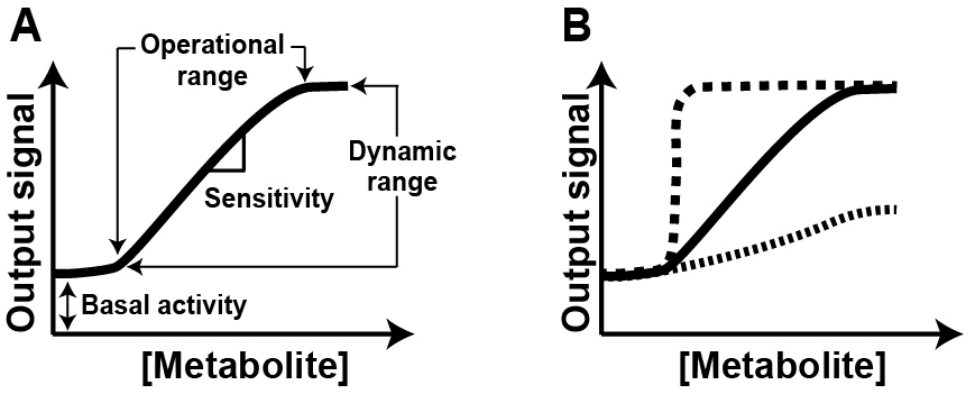

Figure 2.3 Biosensor response curve. (A) The response curve is commonly used to evaluate the performance of a biosensor, and is based on parameters such as the sensitivity and the dynamic- and operational range. (B) A biosensor with high sensitivity can reduce the operational range, resulting in an ON and OFF shaped response curve whereas a low dynamic range often prevents a broad operational range.

2.3 Different types of biosensors

There are different types of genetically encoded biosensors responsive to environmental signals and extra- and intracellular metabolites, and some of these have been simplified and schematically illustrated in Figure 2.4. These biosensors can all be of interest for metabolic engineering applications. For example, although biosensors responsive to environmental signals and extracellular metabolites are of importance, particularly from a bioprocess perspective [71], biosensors responsive to intracellular metabolites have other advantages. For instance, although the final aim in most metabolic engineering endeavours is to secrete the compound or product of interest, secretion often requires additional engineering, for example, finding suitable transporters, which in fact has been shown to be facilitated by using a intracellular metabolite-responsive biosensor [72]. Nonetheless, before dedicating effort for improving the secretion pathway, a proof-of-concept strain must first be developed, which again could be facilitated using biosensors sensing intracellular metabolites. Furthermore, some intracellular metabolites, such as acetyl-CoA and acyl-CoA, are important precursors for producing a wide range of industrially interesting compounds. There is, therefore, also an interest in developing platform strains overproducing these precursor metabolites. Intracellular metabolite biosensors can also be used to sense extracellular metabolites when sensor cells and producer cells are encapsulated in droplets or co-cultivated [73, 74]. Finally, intracellular metabolites are also often the target for balancing metabolism using dynamic pathway regulation.

In this thesis, the focus has been on intracellular metabolite-responsive biosensors based on transcription factors, and before addressing these specifically at the end of this chapter, a brief overview of other relevant biosensors will first be given.

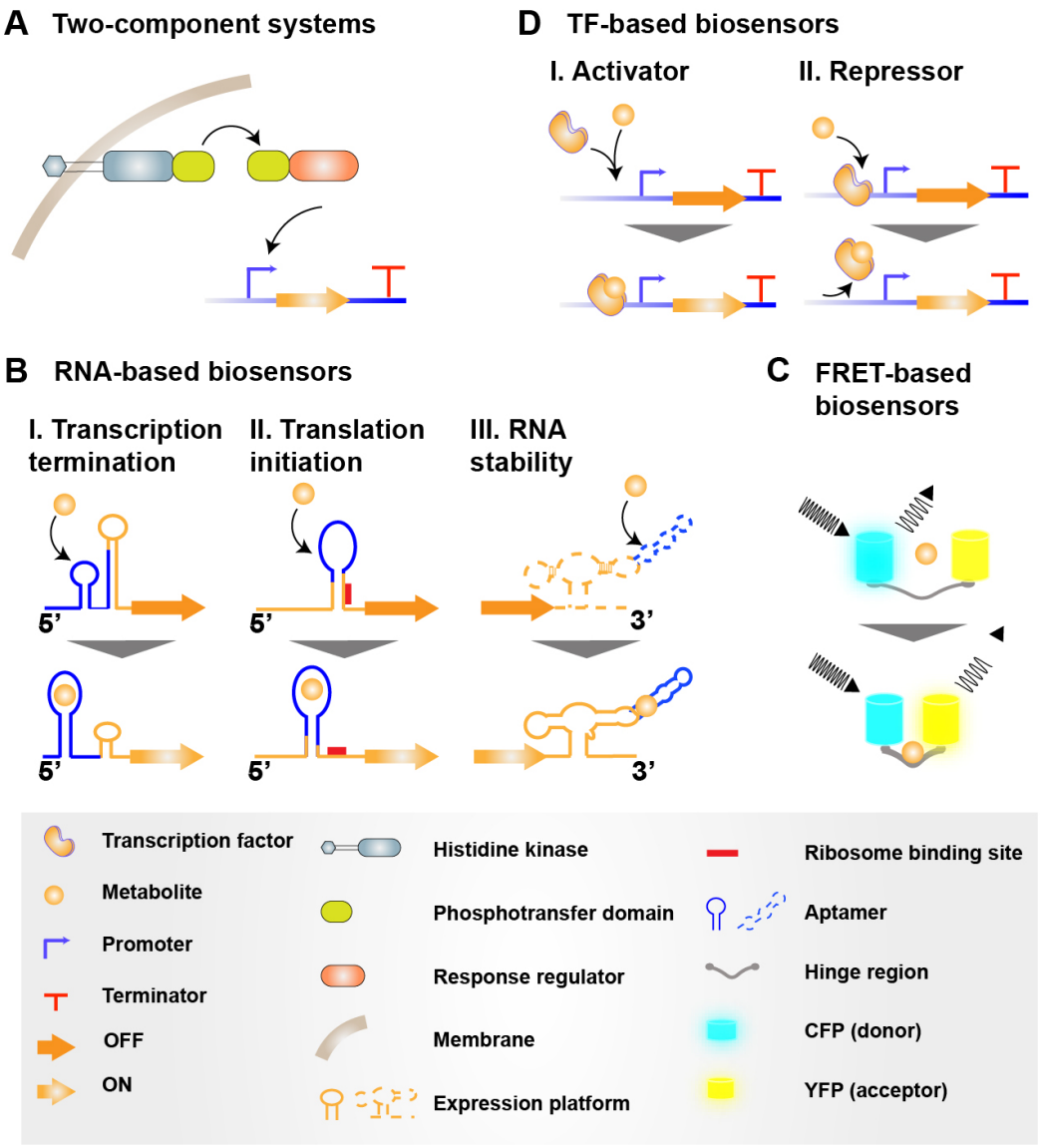

Figure 2.4 Examples of biosensors. (A) Two-component system-based biosensors are mainly composed of a membrane-bound histidine kinase which upon interaction with environmental signals or extracellular metabolites autophosphorylates and in turn phosphorylates a response regulator, which can be a transcription factor regulating gene expression. (B) RNA-based biosensors are mainly based on riboswitches or ribozymes, which are composed of an aptamer domain and an expression platform. RNA-based biosensors can be developed based on the (I) transcription termination, (II) translation initiation level and (III) on self-cleaving ribozymes. (C) FRET-based biosensors can be developed by combining perplasmic binding proteins with fluorescent proteins to achieve fluorescence resonance energy transfer upon ligand binding. (D) Transcription factor (TF)-based biosensors are commonly based on a metabolite-responsive transcription factor and a corresponding promoter to which it can bind and regulate gene expression, either through (I) activation or (II) repression, upon interacting with the metabolite.

2.3.1 Two-component system-based biosensors

Biosensors responsive to environmental signals, for example light, temperature and pH, are commonly based on two-component systems, which are abundant in prokaryotes [53,71]. Two-component systems typically consist of a membrane-bound sensor kinase, a sensor domain connected to an intracellular histidine kinase, and an intracellular response regulator, which often is a transcription factor regulating gene expression. Upon sensing a signal through the sensor domain, the histidine kinase autophosphorylates and in turn phosphorylates the corresponding respone regulator, which either activates or represses gene expression [75] (Figure 2.4A).

The challenge of having a functional two-component-based biosensor is to ensure specific phosphotransfer between the two domains such that off-targeting does not occur [76]. Two-component systems are less present in eukaryotes, which could be due to the more complex systems found in eukaryotes, requiring longer and more stable signaling outputs than can be provided from two-component systems [76, 77]. In eukaryotes, G protein-coupled receptors are one of the most important signaling proteins, which can be used to develop biosensors to respond to extracellular signals and chemicals [78].

For sensing intracellular metabolites, metabolite biosensors are commonly based on proteins, such as metabolite-responsive transcription factors, and regulatory and catalytic RNAs, such as riboswitches and ribozymes.

2.3.2 RNA-based biosensors

A class of metabolite biosensors are RNA-based biosensors, which can be developed from riboswitches. Riboswitches, composed of an aptamer and an expression platform [79], are regulatory elements embedded within an mRNA that can control gene expression at both the transcriptional and translational level by changing its own conformation upon binding to a metabolite [80, 81]. At the transcriptional level, RNA-based biosensors can be used to either disrupt or facilitate the formation of a terminator, resulting in transcriptional activation or repression, respectively (Figure 2.4B). A theophylline responsive sensor was successfully developed using the well-characterized theophylline aptamer, which upon binding to theophylline prevented the formation of an intrinsic terminator, thereby resulting in gene expression [82]. At the translational level, riboswitches can modulate their structure such that the ribosome binding site is sequestered from the ribosome, thus attenuating translation (Figure 2.4B) [83]. Another example of RNA-based biosensors are based on self-cleaving ribozymes, which can be designed to self-cleave in the absence of a metabolite (Figure 2.4B) [84].

What makes aptamers beneficial to use is that, despite their simple structure, they are in many ways protein-like as they can, for example, bind a metabolite of interest with high specificity. There can, however, also be challenges regarding the specificity. As aptamers are chemically less diverse than proteins due to, for example, lack of functional groups, their

metabolite sensing capabilities are less broad. Although existing technologies, such as *in vitro* systematic evolution of ligands by exponential enrichment, can help to increase the functional space of aptamers [85], the challenge lies in identifying aptamers that bind to the target of interest with high specificity and functions *in vivo*. Furthermore, another challenge is to develop functional switches, such that the conformational change caused by a metabolite binding to the aptamer is propagated to the expression platform, generating a readable output. To find a functional switch requires a large number of sequences to be evaluated, and although this can be done *in vitro*, the riboswitches may not be functional in the more complex *in vivo* environment. Using *in vivo*, high-throughput, methods have shown to be promising, but there is still a need to evaluate samples up to 10^{14}, which cannot be easily matched with the currently available high-throughput methods [86]. As aptamers regulate gene expression at transcriptional and translational initiation level, their advantage over protein-based systems is that their regulatory mechanism consumes less energy and cellular resources. Furthermore, riboswitches are not dependent on other proteins and are often encoded on the same transcript as the gene of interest, which can reduce off-targeting [86]. Another type of biosensor with high orthogonality are biosensors based on fluorescence resonance energy transfer (FRET).

2.3.3 FRET-based biosensors

Biosensors based on proteins, for example, periplasmic binding proteins, are another class of biosensors that can be used in metabolic engineering applications. Periplasmic binding proteins are predominantly used in bacteria to sense extracellular molecules, such as carbohydrates, amino acids and ions, and transport them into the cytoplasm [87]. Periplasmic binding proteins consist of two domains connected by a hinge region and exists in an open form and, in the presence of a bound metabolite to the interface of the two domains, in a closed form, bringing the domains closer to each other. By fusing compatible fluorescent proteins, such as cyan fluorescent protein to the N terminus and yellow fluorescent protein to the C terminus of periplasmic binding proteins, fluorescent resonance energy transfer can occur between these two flurophores in the presence of a metabolite (Figure 2.4C) [88]. When the two fluorescent proteins are in close proximity, the energy emitted from the cyan fluorescent protein is taken up by the yellow fluorescent protein, and the energy-transfer efficiency is mesured as a ratio of fluorescence. Other proteins, such as transcriptional repressors, have also been employed to develop sensors based on fluorescence resonance energy transfer, such that upon binding to the metabolite conformational change through the repressor brings the fluorescent proteins closer to allow resonance energy transfer [89].

Biosensors based on fluorescence resonance energy transfer have high orthogonality due to reduced interaction with other host cell components. Despite this, and their ease of construction, their use in metabolic engineering has been limited. This is mainly because of their low dynamic range due to small changes in fluorescence signal. Consequently, they

are not suitable for high-throughput screening, and such sensors can also not be used for dynamic pathway regulation. In contrast, transcription factor-based biosensors are suitable for both dynamic pathway regulation and in high-throughput screening.

2.3.4 Transcription factor-based biosensors

Transcription factors are defined by their ability to regulate gene expression, either positively or negatively, upon binding to a specific DNA sequence, called operator sequence or binding site, on a promoter. Consequently, transcription factor-based biosensors are mainly developed by inserting the binding site of a specific transcription factor into a corresponding promoter, together regulating the expression of a desired gene (Figure 2.4D) [66]. During the last decade, there has been a steady increase in the development and application of transcription factor-based biosensors (Figure 2.5), which have been successfully employed to sense precursor metabolites and compounds of industrial value, such as acyl-CoA [57], malonyl-CoA [58, 90] and muconic acid [91]. It should be noted that during the literature study for Figure 2.5 (see Appendix for listed studies), some relevant papers might have been unintentionally omitted, for which I apologize. This is especially relevant for the year 2000-2010, where great focus was dedicated to the fundamental understanding of synthetic regulatory circuits that were not necessarily developed for metabolic engineering applications. Nonetheless, the trend illustrated here should remain valid, especially the increase after year 2010 [92].

Transcription factor-based biosensors are commonly developed for sensing intracellular metabolites, and this has also been the main focus of this thesis. The coming chapters will focus on these, specifically for metabolic engineering applications in *S. cerevisiae*.

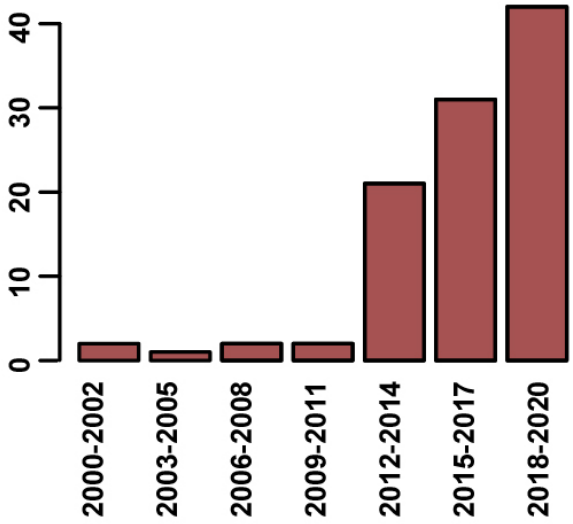

Figure 2.5 Approximate trend of published transcription factor-based biosensor papers. The interest of developing transcription factor-based biosensors for metabolic engineering applications seems to be steadily increasing.

PART TWO

Metabolite-responsive transcription factor-based biosensors

3 | Transcription factor-based biosensors

Transcription factor-based biosensors, here focused on metabolite-responsive transcription factors, are generally divided into two main parts consisting of a sensor and an actuator (Figure 3.1). Overall, there are three components that are of importance, including the metabolite-responsive transcription factor, whose interaction with a corresponding reporter promoter is influenced based on its binding to a specific metabolite. This interaction affects the activity of the promoter, accordingly changing the expression of the reporter gene, for example a gene encoding green fluorescent protein. Ideally, the reporter output should be reflecting the availability of the metabolite by resulting in a corresponding, proportional, output signal (Figure 3.1). In order to suceessfully develop a transcription factor-based biosensor, its main components must be available and match with each other.

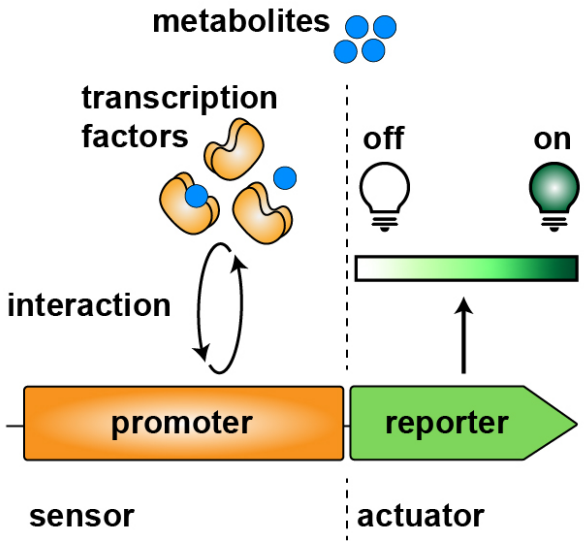

Figure 3.1 Illustration of metabolite-responsive transcription factor-based biosensors. A functional transcription factor-based biosensor is composed of two parts; the sensor part and the actuator part. The sensor part is commonly based on a metabolite-responsive transcription factor and a corresponding promoter with which the transcription factors interact with upon interacting with the metabolites. The actuator part can be defined as a reporter gene encoding a visible phenotype, for example, a green fluorescent protein.

3.1 Transcription factors

Transcription factors are commonly defined as regulatory proteins that are capable of regulating gene expression upon binding to DNA in a sequence-specific manner. Therefore, other proteins essential for gene regulation, for example chromatin remodelers, are not defined as transcription factors due to lack of a DNA-binding domain, making the DNA-binding domain characteristic of transcription factors. There are, however, regulatory proteins classified as transcription factors without having a DNA-binding domain, for example Mga2, a protein involved in regulation of *OLE1* transcription, which will be discussed more in Chapter 5. For metabolite-responsive transcription factors, another characteristic domain is an effector binding domain, which can interact with metabolites [93]. While the effector binding domain is structurally more diverse, the DNA-binding domain is often more conserved among different transcription factors. In fact, based on the DNA-binding domain, transcription factors are classified into different structural groups such as the helix-turn-helix type, the most common DNA-binding domain in bacteria [94].

The majority of metabolite-responsive transcription factor-based biosensors are based on prokaryotic transcription factors (Table 3.1). One reason is that in prokaryotes signal transduction occurs via one-component regulatory mechanisms based on a wide range of allosteric transcription factors, which upon binding to a metabolite results in a conformational change that alters their affinity for their binding sites, thereby regulating gene expression accordingly [77, 95]. Furthermore, prokaryotic transcription factors are advantageous to use in eukaryotes as prokaryotes have a less complex regulatory network, enabling transcription factors, predominantly repressors, to be fairly easily implemented in eukaryotic hosts [96, 97]. Other advantages include their high orthogonality, reducing the risk of interacting with regulatory elements in eukaryotic hosts, and their modularity, allowing the construction of chimeric transcription factors with user-specific functions.

In order to develop a functional and responsive biosensor, it is important that a well-characterized transcription factor is available, and since it is not always straightforward to find a suitable transcription factor, it is common to 're-use' or 're-engineer' an already promising transcription factor for a number of purposes or applications (Table 3.1). From the literature study (Appendix) performed for Table 3.1, it was clear that the majority of studies focused on either demonstrating the potential of transcription-factor based biosensors for metabolic engineering applications or actually implementing well-characterized biosensors for high-throughput screening applications. Although dynamic pathway regulation is another important application, only a few studies focused on dynamic regulation, which is probably due to the complexity of controlling and regulating metabolism on a higher level. Nonetheless, although naturally ocurring sensor systems exist for certain compounds does not necessarily make them "ready-to-use". Commonly, natural sensors need to be custom-made to exhibit, for example, improved dynamic- and operational range [136]. Achieving this can be a straightforward *or* a tedious process, usually requiring the need to at least engineer reporter promoters.

Table 3.1: Examples of metabolite-responsive transcription factors (TFs) used for developing metabolite biosensors.

TFs	Organism[a]	Metabolite[a]	References
AcuR	*Rhodobacter sphaeroides*	acrylate	[98, 99]
AraC	*E. coli*	arabinose	[63, 99–105]
BenM	*Acinetobacter baylyi*	*cis*,*cis*-muconic acid	[72, 91, 106–109]
FadR	*E. coli*	fatty acyl-CoA	[57, 110–113]
FapR	*B. subtilis*	malonyl-CoA	[58, 61, 90, 114–121]
FdeR	*Herbaspirillum seropedicae*	naringenin	[91, 122–124]
Lrp	*Corynebacterium glutamicum*	l-valine	[62, 125, 126]
LysG	*C. glutamicum*	l-lysine	[127–129]
PcaQ	*Sinorhizobium meliloti*	protocatechuic acid	[91, 107, 130]
VanR	*Caulobacter crescentus*	vanillic acid	[107, 130, 131]
XylR[b]	-	xylose	[112, 132–135]

[a]Origin, [b]Origin of organism; several

3.2 Reporter promoters

Promoters are DNA sequences containing regulatory elements to which proteins, including transcription factors, bind to initiate transcription of its downstream gene. Promoter architechtures, especially in eukarytotes, are often complex and can be influenced by several factors, for example by the DNA sequence itself, including the poly(dA:dT) content, the presence of binding sites and other regulatory elements, by chromatin remodeling- and modifying complexes and by the presence of nucleosomes, which are present in varying levels depending on the genes [23, 137].

In eukaryotes, promoters are generally divided into two main parts, the core promoter and the proximal promoter, or the upstream region [138,139]. The core promoter is the region from which transcription is initiated, meaning the region to which the transcription preinitiation complex, including RNA polymerase II and general transcription factors, bind and start transcription. Two features characteristic to the core promoter are the TATA/TATA-like sequences, to which the preinitiation complex is recruited, and the transcription start site, which defines the start of the transcription. Upstream activating sequences or upstream repressing sequences are features found in the proximal region, to which gene-specific transcription

factors bind and regulate gene expression [140] **(Paper III)**.

When developing transcription factor-based biosensors in yeast, it is common to use prokaryotic transcription factors in combination with the host's own native promoter. Therefore, to match these two it is necessary to systematically evaluate binding site positions suitable for the cognate transcription factor. In *S. cerevisiae*, not many promoters have been well-characterized, for example as P_{CYC1} and P_{TEF1}, and even less have been evaluated for use as reporter promoters in biosensor development. Therefore, in **Paper II** we look a bit further into this.

3.3 Reporter genes

The reporter genes are the actuators of biosensors, without which it would not have been possible to monitor any changes taking place in the cell. Commonly, the reporter gene encodes a visible and easily measurable phenotype, including a fluorescent protein or growth-coupled protein that complement an auxotrophic strain or confer resistance to, for example, an antibiotic, allowing the producer cell to be distinguished through improved growth.

Screening based on fluorescence as an output usually requires expensive instruments, whereas selection can be a cheaper alternative and be more useful when screening very large libraries. On the other hand, screens can offer higher resolution, enable less complicated negative/positive selection schemes and may be more suitable for identifying cells producing toxic compounds [50,141]. In this thesis, the gene encoding green fluorescent protein has been the reporter of choice, enabling screening, real-time monitoring and microscopic evaluations.

3.4 Transcription factor-based biosensors for *S. cerevisiae*

S. cerevisiae is widely used in research and industry for several purposes, including as a model organism for studying higher eukaryotes and as a cell factory for producing drugs and chemicals. This is attributed to its well-studied genetics, its ease of engineering, and more specifically for industrial applications, its resistance to phage contamination and the fact that it is generally regarded as safe [142]. Although tools for strain engineering are advancing, development of high-throughput screening tools are lagging behind [33]. Currently, there are only a few well-characterized biosensors available for *S. cerevisiae*, and there is therefore an interest to develop new biosensors or expand on already existing ones, which is the focus of the coming chapters.

4 | Custom-made transcription factor-based biosensors

When initiating the process of customizing a biosensor, it is often assumed that a well-characterized transcription factor is available. As candidate transcription factors often are heterologous, their regulatory function in a chosen host needs to be evaluated together with a modified, endogenous, promoter. Therefore, the first step is to create a transcription factor/promoter pair, which in a subsequent step has to be evaluated for its responsiveness to a desired compound. Finally, the functional and responsive biosensor often needs to be optimized to fit a certain application, for example by improving its dynamic- and operational range.

4.1 Biosensor construction strategies

To create the transcription factor-promoter pair, different binding site positions and their influence on promoter activity must be evaluated. This is mainly because promoters are complex, knowledge of their detailed structure is still lacking and the presence of other important elements are yet to be elucidated [143]. Furthermore, sequence variations as short as 2 bp, especially in the core promoter, can influence promoter activity, and sequences immediately upstream of the translation start site can affect translation efficiency [144]. As transcription factor-based biosensors developed for *S. cerevisiae* are commonly based on prokaryotic transcription factors, mainly repressors, the binding sites are located in the core promoter in order to achieve repression through steric hindrance of RNA polymerase progression or binding of the preinitiation complex [66]. One approach is to place the binding sites either close to the TATA box or the transcription start site, which have shown to work well previously by allowing for repression and/or activation depending on the sensor-type [58, 61, 91]. Although this is a convenient approach, the drawback is often reduced promoter activity, which could, for example, be due to sequence variations caused by the inserted binding sites [144]. This reduced promoter activity is not desirable, particularly when using repressors, as the maximal dynamic range is reduced. To find locations that have minimal influence on promoter strength, one usually has to evaluate several locations.

The next step is to ensure responsiveness to the compound of interest. This is usually performed by feeding or inducing the production of the desired compound and evaluate

changes in the output signal. Here, one will obtain better understanding of the operational range, and ideally there should be incremental changes in output signal as a response to a broad range of concentrations that are of relevance to the application. The response to a compound is also dependent on the expression level of the transcription factors, which can be modified using inducible promoters or promoters of different strengths.

We were interested in obtaining a better understanding for how to facilitate the construction and development of transcription factor-based biosensors. We sought to improve the dynamic range of a previously characterized malonyl-CoA responsive biosensor **(Paper II)**. We also discuss the possibility of using the CRISPR/Cas9 system for better understanding of promoter architecture and guiding binding site positioning **(Paper III)**.

4.1.1 Malonyl-CoA-responsive biosensor - an example case

Malonyl-CoA is an important signaling molecule in living cells and the precursor for producing many essential compounds such as fatty acids and flavonoids as well as the industrially interesting chemical 3-Hydroxypropionic acid. There is therefore an interest in further developing and optimizing a previously developed malony-CoA-responsive biosensor, which is based on the bacterial transcription factor FapR (**f**atty **a**cid and **p**hospholipid biosynthesis **r**egulator). FapR is a malonyl-CoA-responsive transcriptional repressor derived from *Bacillus subtilis*, where it negatively regulates its own expression and the biosynthesis of fatty acids and phospholipids when malonyl-CoA levels are low [145, 146]. To date, a malonyl-CoA-responsive biosensor based on FapR has been employed in several different organisms [67], including in bacteria [120], yeast [58, 61] and mammalian cells [118].

As a first step to improving the maximal dynamic range of the malony-CoA sensor, we were interested in evaluating the importance of binding site locations in different promoters. Only a few promoters such as P_{CYC1} [91], P_{TEF1} [58], P_{GPD1} [61], P_{GPM1} [134] and hybrid promoters based on P_{GAL1} [110, 113, 117, 135, 147] have been characterized and employed for constructing biosensors in *S. cerevisiae*. Therefore, in **Paper II** we characterized additional promoters that might be suitable for constructing biosensor that can be employed in both high-throughput screening applications and dynamic pathway regulations. For instance, for high-throughput screening, a dynamic range as high as possible might be required to distinguish strains with different performances whereas for dynamic pathway regulation a suitable range is determined by the optimal expression level of the involved genes. Our first immediate finding corroborates the generally accepted fact that promoter architectures are complex and that there is still much we do not know about important elements embedded within these [148]. This could be observed in the different outputs we achieved despite placing the same binding site in areas of, more or less, equal importance, for example, close to the TATA box, the transcription start site and between these two **(Paper II)**.

For example, what we found interesting was that insertion of binding sites placed in different locations in P_{MDH2} were all mainly unresponsive to FapR. Of these, two inserted

binding sites resulted in very low fluorescence signals, suggesting that these regions play a role in promoter activation (Figure 4.1A). On the other hand, all binding sites located in P_{CCW12} seemed to be responsive to FapR, although binding site 2, which did not influence the promoter activity, gave a strong repression in presence of FapR, reducing the leakiness and resulting in a high maximal dynamic range (Figure 4.1B). It is also interesting that binding site 1 in P_{CCW12} is 6 bp upstream of the TATA box, whereas binding site 2 is 4 bp downstream of it, and yet a clear difference in promoter activity and repression can be observed. Furthermore, for P_{CCW12} we also observed that the basal activity in the OFF state, that is in the presence of the transcription factor, increased the further away from TATA box and closer to the transcription start site the binding site is. Perhaps steric hindrance is more prominent when the presence of transcription factors prevents the binding of the preinitiation complex, that is when they bind in close proximity to the TATA/TATA-like elements, and have less influence in hindering RNA polymerase II from initiating transcription. Most yeast promoters contain several transcription start sites with one site usually being the more dominant one. If this particular site would be masked, RNA polymerase could potentially scan for other transcription start sites, and thereby proceeding with the transcription.

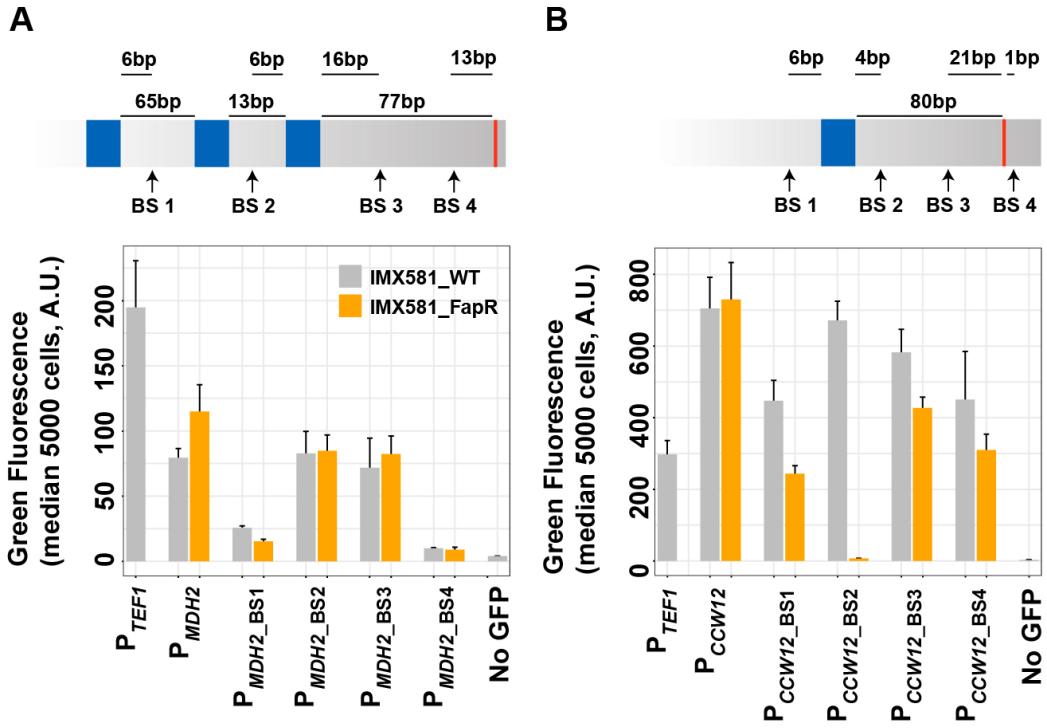

Figure 4.1 Evaluating *fapO* binding site positions in P_{MDH2} and P_{CCW12}. *FapO* binding sites were placed in the proximity of the TATA box and the transcription start site (TSS) in the core promoter of (A) P_{MDH2} and (B) P_{CCW12}. The reporter promoters were placed upstream of GFP and the fluorescence was measured 8 h and 24 h after inoculation for P_{CCW12} and P_{MDH2}, respectively, using flow cytometry. The blue boxes indicate TATA box and the red line represents the TSS. n = 3, error bar = ± SD. Adapted from [115].

The other promoters we studied, P_{TDH3}, P_{TPI1} and P_{ADH2} (**Paper II**), overall showed the same pattern, suggesting that the area where the TATA or TATA-like element is located is a hotspot for achieving desirable, conditional, expression. This pattern could also be observed in other studies [107,148]. In a recent study, binding site positions of the transcription factor VanR was thoroughly evaluated in the area between the TATA box and TSS in P_{TEF1}, showing that the basal activity in the OFF state increases the further downstream from the TATA box the inserted binding site positions are, thereby resulting in increased leaky expression. Despite these agreeing patterns, a universal rule cannot be claimed. This is not only due to the complex nature of promoters but also because the binding site length and the transcription factor type can influence the expression level by, for example, altering the sequence composition and sterically hindering binding of other regulatory proteins to different degrees based on the shape and size of the transcription factor. These suggestions can, however, be used as guidelines and as starting points for finding promising locations that results in a desirable dynamic range.

Another type of reporter promoters that are not commonly used are inducible promoters. In our study (**Paper II**), we also included a glucose-inducible promoter, namely P_{ADH2}, which is strongly induced when glucose is depleted [149]. Using P_{ADH2} in dynamic control can enable the separation of growth phase from production phase, and adds two layers of regulation; the absence of glucose and the presence of accumulated malonyl-CoA levels to derepress gene expression. This design can, for example, reduce potential leaky expression and provide more controlled regulation [113, 150].

In order to improve the maximal dynamic range for repressor-based biosensors, the influence of the binding sites on promoter activity should ideally be minimal and the basal activity in the OFF state should be as low as possible. As finding promising binding site locations and preventing the promoter activity from decreasing upon binding site insertion can be challenging, we sought to evaluate whether we could re-arrange the positions and yet achieve a functional sensor. We decided to use P_{TEF1}, a commonly used reporter promoter for repressor-type biosensors, and placed the binding sites in the upstream region, outside of the core promoter (Figure 4.2A). Here we observed that the closer the binding sites (binding sites 5 and 6) were to the core promoter and the TATA-like element, the more the promoter activity was reduced. On the other hand, binding sites placed further upstream had neglible effect as the promoter activity was not compromised (**Paper II**).

As the majority of bacterial repressor function in yeast through steric hindrance, we assumed that expression of FapR alone, when bound to the binding sites in the upstream region, would not result in repression. Therefore, we fused FapR to the endogenous repressor Mig1, creating a chimeric transcription factor (Figure 4.2A). Mig1 is a zinc finger protein known to play a key role in glucose repression, repressing a number of genes in the presence of glucose [151]. Like a number of other repressors in yeast, including Rox1, Mig1 is thought to function by recruiting the Tup1-Ssn6(Cyc8) corepressor complex [93, 152]. When evaluating both Mig1 and Rox1, we found that Mig1 exhibits stronger repression (**Paper II**), and

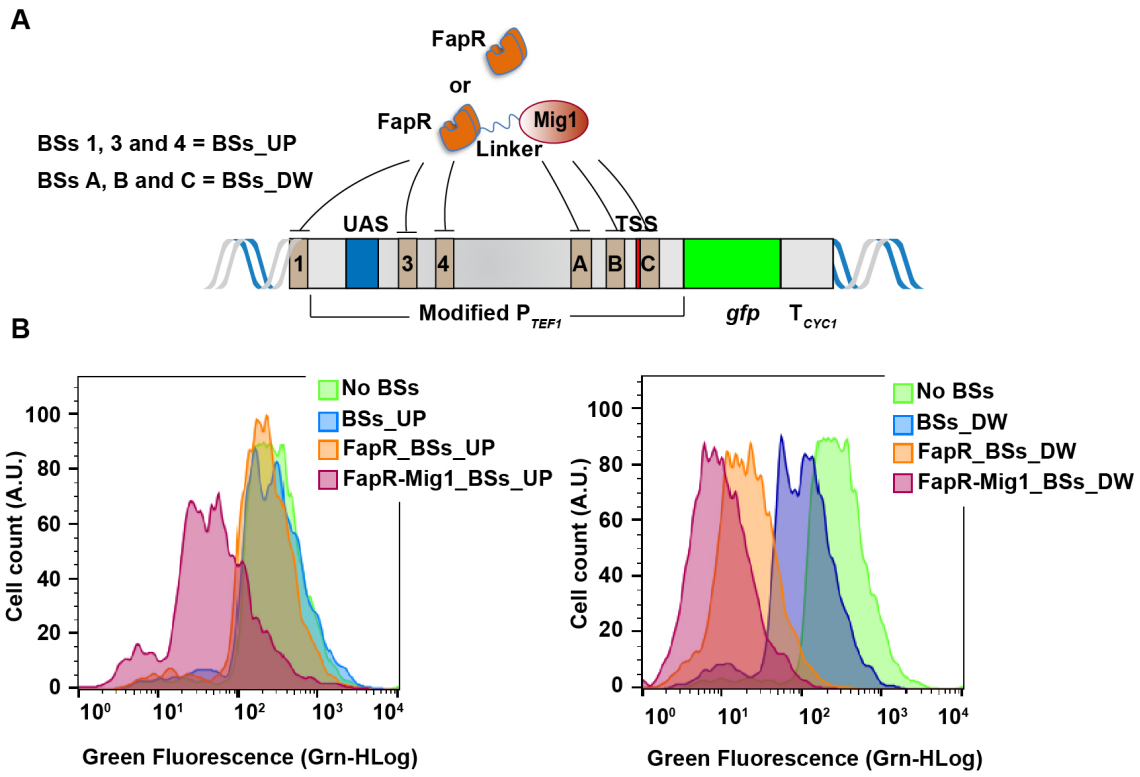

Figure 4.2 Chimeric transcription factor improves dynamic range. (A) Design of inserted *fapO* binding sites in both the upstream region (1, 3 and 4) and the downstream region (A, B and C) in P_{TEF1}, which were evaluated with FapR as well as with FapR-Mig1. (B) Histograms showing the influence of binding sites inserted in the upstream- and in the downstream region and the achieved repression using FapR and FapR-Mig1. Fluorescence was measured 8 h after inoculation using flow cytometry. n = 3, error bar = ± SD. Adapted from [115].

decided therefore to continue with it. The responsiveness of FapR to malonyl-CoA was maintained in the chimeric transcription factor, indicating that fusion with Mig1 did not negatively influence its responsitivity (**Paper II**).

Combining the binding sites in the upstream region did not alter promoter activity, whereas binding sites combined in the downstream region resulted in decreased promoter activity (Figure 4.2B). This data suggest that he maximal dynamic range can be improved by re-positioning the binding sites to the upstream region and using a chimeric transcription factor. Furthermore, the chimeric repressor was shown to be promising to reduce leaky expression when combined with the downstream binding sites (Figure 4.2B). Although using Mig1 appears to be a good choice for such purposes, it is important to keep in mind that it is a ubiquitous protein and might interfere with other processes in the cell, which might not be ideal for cell factory development. Similar challenges could occur using other yeast repressors, such as Ssn6 [133], due to the highly interconnected nature of yeast transcription factors.

In order to more efficiently find promising areas within reporter promoters for biosensor construction, it is necessary to find other means to evaluate promoters in contrast to the systematic analysis of binding site positions. Using CRISPR-mediated gene expression activation (CRISPRa) could potentially be used to 'pre-screen' promising areas in promoters.

4.1.2 Using CRISPR to facilitate biosensor development

The CRISPR/Cas system is a defense mechanism involved in phage immunity in many bacteria [153], and has received considerable attention for its application in genome engineering [154, 155]. In fact, its discovery has been of such importance that it was awarded the Nobel Prize in Chemistry in 2020. There are different types of CRISPR systems [156], and the one being mainly employed for genome editing and gene regulation is based on the type II system from *Streptococcus pyogenes*, which uses a single Cas protein, Cas9, together with a single guide RNA (gRNA) [39, 157]. A catalytically inactive Cas9, often referred to as dCas9 (endonuclease-deficient Cas9) [158, 159] has been widely used as a programmable tool for gene regulation in CRISPRi/a applications [160, 161].

In **Paper III**, we evaluate the interplay between dCas9, fused to the tripartite VPR composed of the three transcriptional activators VP64, p65 and Rta, and transcription factor binding sites of Gcr1, Gcr2 and Tye7 in 10 different glycolytic promoters. Gcr1, Gcr2, Tye7 are all extensively studied transcriptional activators in yeast. Gcr1 and Gcr2 act as heterodimers with Gcr1 carrying the DNA binding domain and Gcr2 the activating domain. The Gcr1/Gcr2 heterodimer binds to the consensus motif GGAWGC, and has most of its targets identified in the glycolytic pathway [162, 163]. Tye7 is a basic helix-loop-helix transcriptional activator with the consensus binding motif (CAT)CACGTG, and most of its targets are also identified in the glycolytic pathway [164]. The promoters used in our study (**Paper III**) have known binding sites for Gcr1/Gcr2 and Tye7. In order to study the interplay, or potential competition, between dCas9-VPR and the transcription factors Gcr1/Gcr2 and Tye7, the promoters were coupled to the gene encoding green fluorescent protein and integrated into the genome of *S. cerevisiae*. To study the effect of dCas9-VPR binding, gRNAs were designed to either bind on top or outside of a known motif. For controls, we designed gRNAs to bind somewhere 'random' in the core promoter or in the proximity of it. When evaluating the gRNAs designed to bind on top of a motif, we found that the fluorescence signal either decreased or remained unchanged compared to the control (Figure 4.3). On the other hand, placing the gRNA outside of a motif resulted in increased GFP signal, indicating that there is a synergistic effect when the dCas9-VPR is not bound to a motif whereas a competitive effect is possible when dCas9-VPR binds on top of a motif.

While these results could be of fundamental interest, it is also worth noting that such data can be used to direct the design of gRNAs for CRISPRi/a. In addition, it could also provide information on the importance of specific transcription factor binding sites for gene regulation. However, the hypothesis derived from our study that dCas9 most likely competes with the

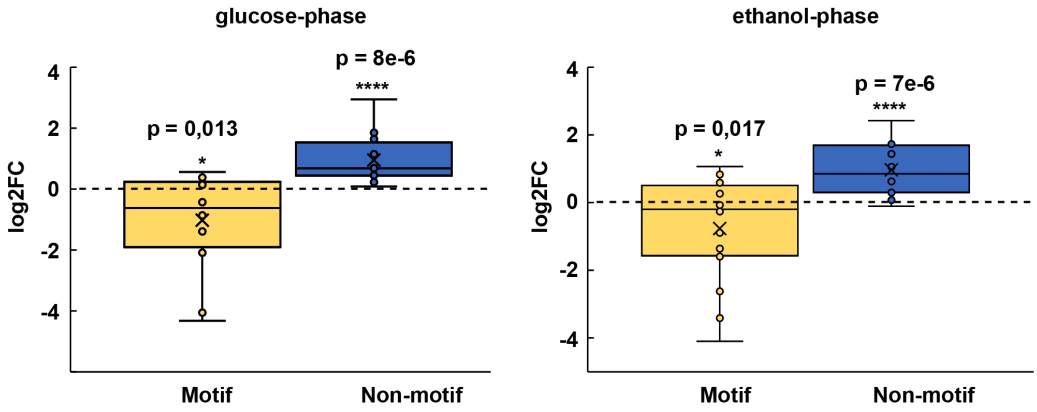

Figure 4.3 Targeting dCas9-VPR to a transcription factor motif or non-motif position. gRNAs were designed to target motif or non-motif positions bound by either Gcr1, Gcr2 or Tye7 within 10 glycolytic promoters, which were placed upstream of GFP. Fluorescence measurements were done using Biolector. n = 3, error bar = ± SD. *p-value <0.05, **p-value <0.01 (Student's t test).

endogenous transcription factor when bound on top of a motif could potentially have been confirmed through further studies and additional control experiments. For example, the same set up could have been run with only dCas9 without the activator VPR. Or, the binding sites for the transcription factors Gcr1, Gcr2 and Tye7 could have been mutated/deleted to allow for comparison between conditions in the presence and absence of the transcription factor binding. Another option would be to perform chromatin immunoprecipitation experiments to demonstrate transcription factor occupancy.

This paper is not directly related to biosensors, but there are some aspects of it that could be interesting for biosensor development. For example, when evaluating the effect of the control gRNAs designed to bind to unspecific positions within the promoter, which to our knowledge does not contain any known binding sites, we observed different output signals. This indicates that there might be important elements in these areas that are yet to be elucidated. For biosensor development, such strategy might facilitate developing synthetic promoters and help guiding binding site positioning. For example, if there are areas within the promoter that result in a clear negative or positive fold change upon expression of dCas9-VPR, then perhaps this area is interesting to look further into. Such areas could indicate the presence of important regulatory factors, which could be a useful area for placing a repressor-type transcription factor as there is a possibility of temporarily interaction with a regulatory element. Using dCas9-mediated gene regulation might not necessarily be comparable with the results obtained when evaluating binding site positions with a corresponding transcription factor, but it could provide a better understanding of promoter architechture. This is especially relevant as the benefit of using dCas9 as a pre-screening tool is not dependent on any specific binding sites, the length of these and their interaction with transcription factors, and might thereby give a more general overview.

PART THREE

Sensing fatty molecules

5 | Fatty acid-responsive biosensors

Fatty acids are involved in several important processes in living cells, including as signaling molecules, as energy storage, in cell membrane formation and in protein modification processes (Figure 5.1) [165,166]. Although fatty acids are essential molecues in living systems, the acids are usually not found in a free state and serve several important roles when in combination with other molecules. One important function is storing energy in the form of triacylglycerols or steryl esters. Another function is as building blocks of phospholipids and glycolipids, which are important components of biological membranes. Furthermore, fatty acids also serve as hormones and intracellular messengers and play a role in localizing certain proteins to the membranes. In addition to their physiological roles, fatty acids and fatty acid-derived compounds, including fatty alcohols and alkanes, are also of great interest for industrial applications [167]. These can be used to produce biofuels and several valuable chemicals and pharmaceuticals, reducing the need of petroleum-driven production (Figure 5.1) .

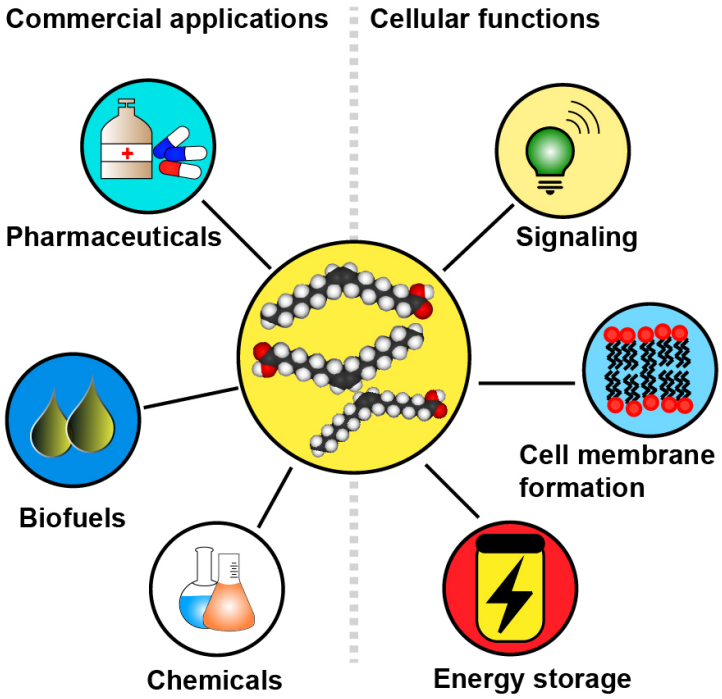

Figure 5.1 Functions and applications of fatty acids. Fatty acids have several important cellular functions as well as also being of industrial interest.

5.1 Fatty acids as industrially relevant compounds

Fatty acids and fatty acid-derived products have a wide range of applications, making them industrially attractive to produce at commercial levels [168]. For example, oils and fats derived from plants and animals are referred to as oleochemicals and include fatty acids, fatty alcohols, fatty acid methyl esters and fatty amines, which can be used in products such as soaps and detergents, biofuels, cosmetics, lubricants and nutraceuticals [167]. The most common source for production of today's oleochemicals comes from plant oils, including palm and soybean. There are, however, numerous challenges of such production. First, desired oleochemicals with certain properties [169, 170], including chain length and saturation, may not be abundant in such plants, and in order to obtain oleochemicals with desired composition requires rarer plants [171]. The challenges of using rare plants includes low productivity and the need to cultivate these in a specific climate and soil condition, making it unreliable in terms of flexibility and stability and economically infeasible. The need for a certain climate is also true for cultivating palm and soy, which has resulted in environmental and ethical problems due to increased deforestation and destruction of important habitats.

Therefore, it has become increasingly important to find alternative ways to produce oleochemicals to replace petrochemicals, and one potential way is through microbial cell factories. Some microorganisms referred to oleaginous yeast, including species of the genera *Rhodosporidium* and *Yarrowia*, are natural producers of high levels of lipids [172]. However, since these microorganisms do not have the same advantage as *S. cerevisiae* in terms of engineering tools, knowledge and databases, they are not as well-established. Therefore, despite the fact that *S. cerevisiae* is a naturally low producer of lipids, in fact only 2-3% of its cell dry weight is composed of lipids, its metabolism has been successfully engineered to produce a wide variety of fatty acids and derived products, although still not at a commercially relevant level. To be able to engineer the metabolism for fatty acid production, it is important to have an understanding of the metabolism, particularly the fatty acid metabolism. Since this topic is beyond the scope of this thesis, a brief overview of *S. cerevisiae* fatty acid metabolism will be provided and following this different engineering strategies will be discussed to exemplify the challenges of engineering strategies.

5.2 Fatty acid metabolism in *S. cerevisiae* - a brief overview

As fatty acids are involved in several different processes, it is essential to maintain homeostasis. *De novo* synthesis of fatty acids are carried out by the **f**atty **a**cid **s**ynthase (FAS) complex. There are two types, type I FAS and type II FAS, which are independent from each other and function in the cytoplasm and mitochondria, respectively [173, 174]. The mechanisms between these types are quite different as the type II FAS, which is commonly found in prokaryotes, is composed of individual and separate enzymes whereas type I FAS consist of highly integrated enzymes functioning within a multi-enzyme complex, encoded by the genes *FAS1* and *FAS2*. The precursor for all fatty acids is acetyl-coenzyme A (acetyl-CoA),

which is carboxylated to malonyl-CoA through acetyl-CoA carboxylase, encoded by *ACC1* (Figure 5.2). This reaction is irreversible and thus the commited step for fatty acid synthesis.

The FAS complex catalyzes the condensation of acetyl-CoA and malonyl-CoA to acyl-CoA, which is the main effector molecule regulating expression of genes involved in the fatty acid metabolism [175]. The fatty acid spectrum consists of C_{12}-C_{26} fatty acids, where the majority consists of long-chain fatty acids, C_{16} and C_{18}. These serve as precursors for production of very long-chain fatty acids, C_{20}-C_{26} fatty acids, carried out by the elongases Elo2 and Elo3 on the endoplasmic reticulum (ER) surface. Fatty acids can be unsaturated through the single enzyme system Ole1, a Δ9-fatty acid desaturase [176, 177]. In fact, the majority of fatty acids in *S. cerevisiae* are unsaturated, $C_{16:1}$ and $C_{18:1}$ [177]. Since fatty acids are of fundamental, medical and industrial interest, there has been great amount of research aiming to understand its metabolism, for example, for producing fatty acid and fatty acid-derived products.

5.2.1 Engineering fatty acid metabolism

As mentioned in previous chapters, engineering microorganisms to produce industrially relevant compounds, including fatty acids, often requires major re-programming of metabolism. Commonly, the metabolism has to be rewired on several different levels in parallel in order to find an optimal route [168]. For example, the central carbon metabolism needs to be engineered to enhance the precursor supply (Figure 5.2). Since acetyl-CoA is an essential building block for producing a number of different compounds and the major substrate for the fatty acid synthetase, it is of great interest to increase the carbon flux towards acetyl-CoA [178]. Although acetyl-CoA is synthesized in four different compartments, including the mitochondrion, peroxisome, nucleus and the cytosol, it cannot be transported across membranes unless certain shuttle mechanisms are in place. There is, however, an interest to transport acetyl-CoAs from other compartments to the cytosol to circumvent acetyl-CoA from being generated from pyruvate via the cytosolic reactions (Figure 5.2). This is due to the Crabtree effect, which is that *S. cerevisiae* has evolved to use fermentative metabolism despite the presence of oxygen when glucose is in excess [179]. Briefly, this results in less ATP production and increased ethanol fermentation. In a recent study to reprogram *S. cerevisiae* into a fatty acid-producing yeast, several approaches, including rational engineering and directed evolution, were taken to balance pathway intermediates, meet the NADPH demand and ensure viability despite abolishing ethanol fermentation, the preferred route to produce energy molecules in presence of glucose [45]. Other approaches includes overexpressing the *ACC1* activity [180], deleting the beta-oxidation pathway [181], and various other strategies such as engineering the FAS complex to increase production of short- and medium chain fatty acids [182] and to improve the production of very long chain fatty acids using a heterologous FAS I system and rewiring the native fatty acid elongation system [183]. These approaches usually require laborious and time-consuming genome-scale engineering strategies.

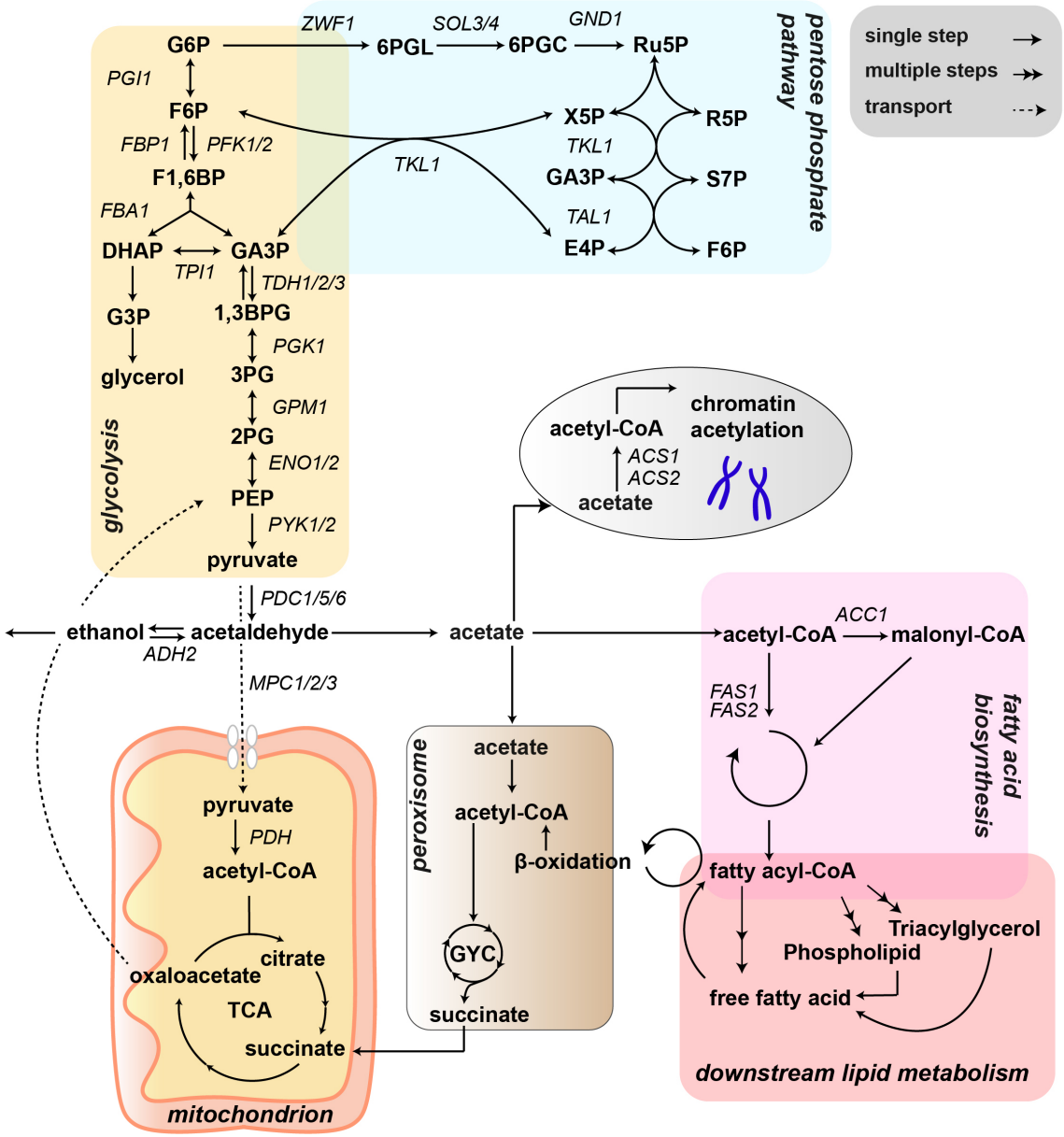

Figure 5.2 Simplified overview of some key pathways in the metabolic network. Due to the highly interconnected and dynamic nature of metabolism, genome-scale engineering is often required to improve the carbon flux through a certain pathway, for example to improve fatty acid production.

An alternative or complementary approach to genome-scale engineering would be the use of fatty acid-responsive biosensors as these have proven to be useful in improving production of fatty acid-derived products through dynamic pathway regulation [57] and in finding genes not previously known to enhance the levels of fatty acids and derived products (**Paper IV**).

5.3 Fatty acid-responsive biosensors and their applications

As is the case of constructing any kind of transcription factor-based biosensor, it is necessary to have a well-characterized transcription factor responsive to your compound of interest as a starting point. There are several naturally existing transcription factors that are responsive to certain fatty acids, which potentially can be employed to develop fatty acid-responsive biosensors. Here, the interest has been on developing biosensors responsive to long-chain fatty acyl-CoAs, C_{16} and C_{18}, as these are the main fatty acid components, using transcription factors mainly derived from prokaryotes.

5.3.1 Screening for genes enhancing fatty acyl-CoA levels

A well-characterized transcription factor responsive to long-chain acyl-CoAs is FadR (**F**atty **a**cid **d**egradation **r**epressor) from *E. coli* [184, 185]. FadR has been reported to have a dual function as it can act as a positive regulator of genes involved in the biosynthesis of unsaturated fatty acids and as a negative regulator of genes involved in fatty acid transport and degradation through beta-oxidation [184]. FadR is released from its binding sites in the presence of fatty acyl-CoAs as binding to these results in a changed conformation, weakening its binding to DNA. A biosensor based on FadR has previously been developed and employed to improve fatty acid-derived products in *E. coli* [57], demonstrated to work in *S. cerevisiae* [110] and applied in our study in high-throughput screening to identify genes boosting fatty acyl-CoA levels (Figure 5.3) (**Paper IV**).

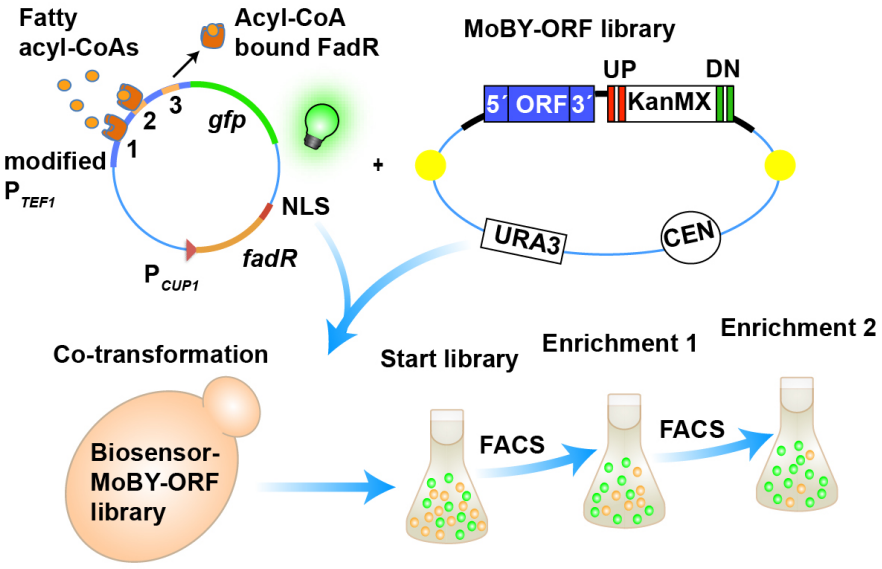

Figure 5.3 Overview of the FadR-based screening strategy. FadR-based sensor was co-transformed with the MoBY-ORF library, and cells exhibiting increased GFP signal were sorted through two enrichment steps using fluorescence-activated cell sorting (FACS). Adapted from [111].

As the fatty acid metabolism is highly dynamic, we assumed that there must be genes not previously known to influence the fatty acid metabolism, and we were interested to evaluate whether such candidate genes could be identified with the FadR-based sensor. Therefore, we combined the sensor system with the MoBY-ORF library, a gene overexpression library containing 4956 uniquely barcoded open reading frames of *S. cerevisiae* [186], to screen for genes enhancing the fatty acyl-CoA pool. One immediate challenge with such an approach is that the target metabolite, fatty acyl-CoA, is an endogenous compound that is highly, and tightly, regulated. Such regulation can therefore prevent accumulation of fatty acyl-CoAs, thereby making the screening challenging. Despite this, the screening was carried out employing fluorescence-activated cell sorting, and the populations resulting in increased fluorescence signal were analyzed by sequencing the gene-specific barcodes in order to identify the genes responsible for an increased output. For the enriched cells, we did not have any strict cut-off, and decided to instead analyse single genes that were either found to be highly enriched, enriched in both sorting steps or enriched only in the first sorting (Figure 5.4).

We decided to evaluate the 16 genes marked in Figure 5.4 by measuring both the fatty acid levels and the fatty alcohols when the genes were overexpressed in a strain expressing an acyl-CoA reductase. From these measurements, we found three candidate genes of interest, including *GGA2*, *LPP1* and *RTC3* as these led to increased fatty alcohol levels (Figure 5.5) (**Paper IV**). The gene *RTC3* was particularly interesting as its overexpression resulted in not only increased fatty alcohol levels but also changed composition with significantly increased levels of C_{18}. The results observed from overexpressing *RTC3* are, however, unclear, especially as there has not been much research on this protein and the fact that it has been reported to be involved in a number of different mechanisms [187, 188]. The gene encoding Gga2, which seems to have a better understood mechanisms than Rtc3, was also promising as it resulted in increased levels of both fatty acids and fatty alcohols (Figure 5.5). However, it is still unclear how overexpression of *GGA2* resulted in increased fatty acid and fatty alcohol levels, but one hypothesis is that it through direct or indirect interaction with lipid components, including phosphoinositides and inositol, results in increased gene expression of fatty acid biosynthetic genes [189–192]. On the other hand, Lpp1 seems to be the one that has the strongest connection to the lipid metabolism since it is involved in regulating phospholipid metabolism through dephosphorylation of diacylglycerol, which can be converted to triacylglycerol and through subsequent steps be activated to acyl-CoAs (Figure 5.2) [193].

The overall screening could have been more accurately analysed by including an additional, constitutively expressed, fluorescent protein such as mCherry to be used for normalization [194]. A constitutively expressed fluorescent protein would reflect a cell's overall transcriptional capacity and thereby enable a more accurate representation of all metabolite-responding cells including cells with low GFP fluorescence caused, for example, by growth defects or other intracellular interferences as a consequence of overexpressing certain genes.

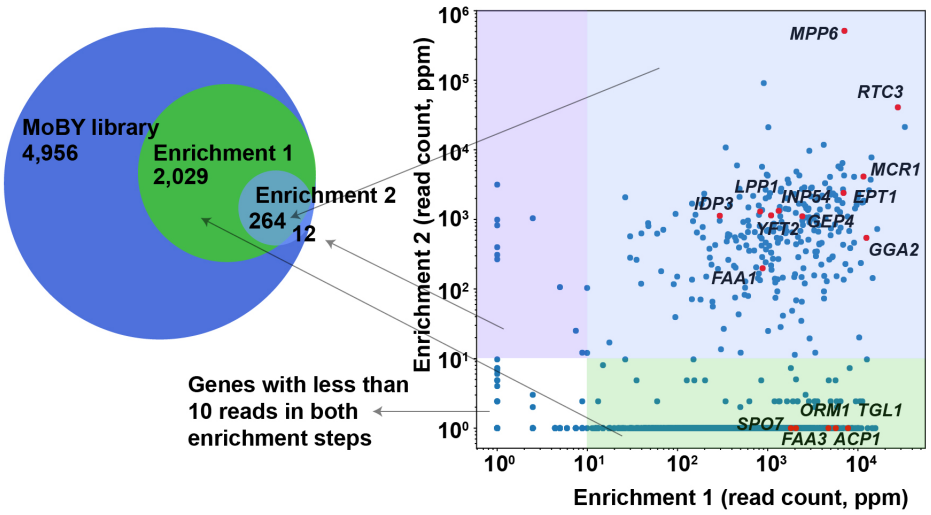

Figure 5.4 Library analysis. From the MoBY-ORF library, which contains 4956 uniquely barcoded open reading frames of *S. cerevisiae*, two subsequent enrichment steps of strains with increased fluorescence were performed using a fatty acyl-CoA responsive biosensor and fluorescence-activated cell sorting. Candidate genes (red) were choosen from both the first- and second enrichment step for further evaluating their fatty acid and fatty alcohol producing capability. For each gene, the reads were normalized to the total number of reads in the library (in ppm). Adapted from [111].

5.3.2 Saturated fatty acid-responsive biosensors

Another transcription factor responsive to long-chain fatty acyl-CoAs is FabR (**F**atty **a**cid **b**iosynthesis **r**epressor), also derived from *E.coli* [195,196]. The difference between FabR and FadR is that FabR is mainly responsive to the saturated/unsaturated ratio and does not have a dual function as FadR. A transcription factor with similar properties to FabR is DesT from *Pseudomonas aeruginosa* [197–199]. We were interested in developing a dual-biosensor based on a combination of either FadR with FabR or with DesT, depending on the one performing the best. We were also interested in evaluating another variant of FadR derived from *Vibrio cholerae* as this has been reported to have higher affinity for long-chain fatty acyl-CoAs [200]. The idea was to ultimately have a method enabling a more specific sensing of the saturated or unsaturated levels. There has not been much research on these transcription factors, and neither have these previously been employed for biosensor development. Therefore, the first step was to evaluate their potential as metabolite biosensors.

While evaluating the bacterial transcription factors, we were also interested in developing an endogenous saturated fatty acid-responsive biosensor based on the promoter P_{OLE1}, which we placed upstream of GFP (Figure 5.6). The idea was to used this as a control for real-time measurements of the changes in the saturated/unsaturated fatty acid ratio throughout the different growth phases, and thereby using it when evaluating the responsiveness of the biosensors based on the heterologous transcription factors FabR and DesT. To evaluate this sensor system, we expressed it from a centromeric plasmid in both the wild type-strain

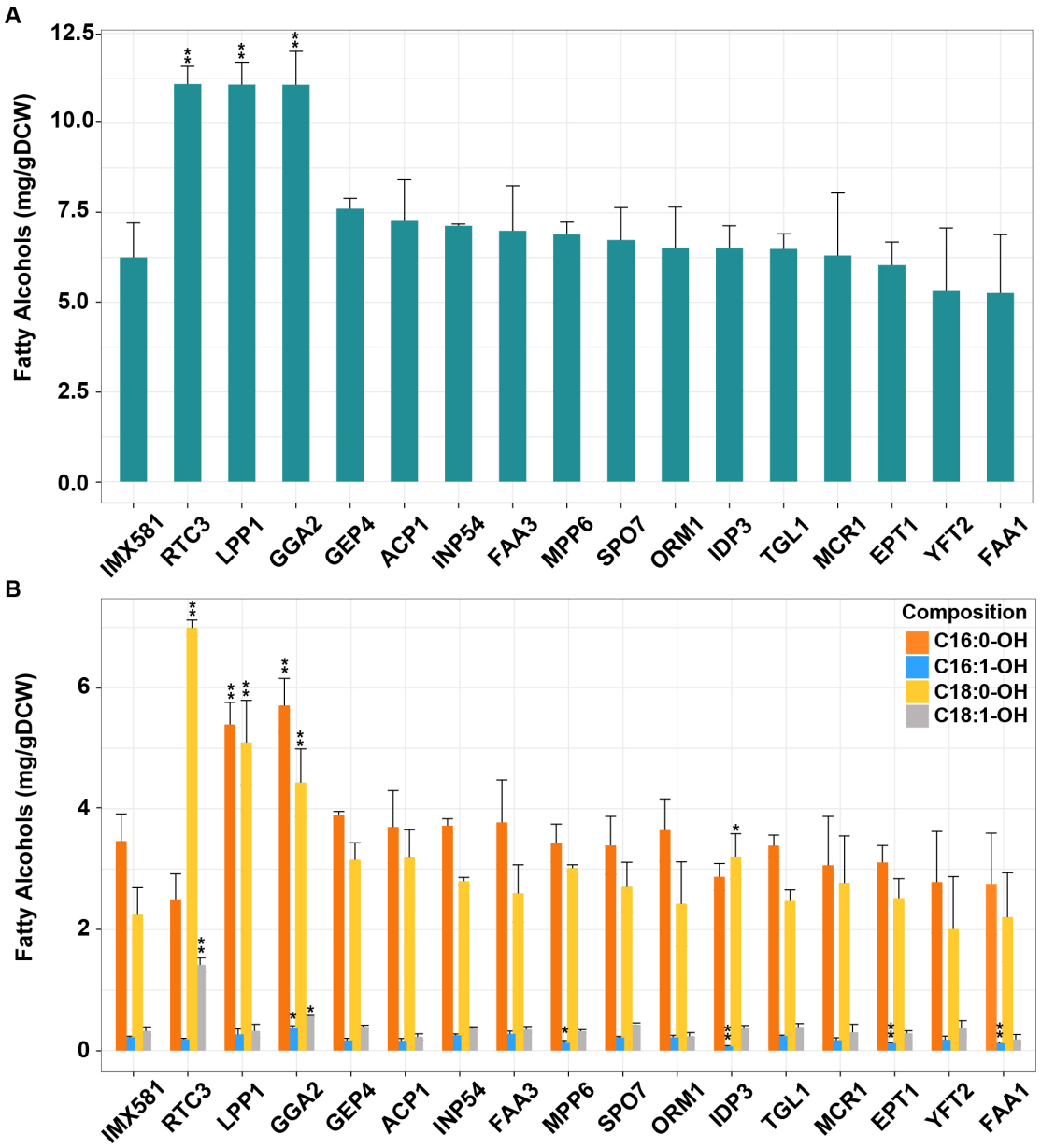

Figure 5.5 Fatty alcohol production analysis. (A) The total fatty alcohol production and (B) the fatty alcohol composition was analysed for strains overexpressing the respective candidate genes and containing a fatty acyl-CoA reductase. The control strain is the background strain IMX581, in which candidate genes and the fatty acyl-CoA reductase were overexpressed. Samples were harvested 72 h after inoculation. n = 3, error bar = ± SD. *p-value <0.05, **p-value <0.01 (Student's t test). Adapted from [111].

CEN.PK113-11C and a modified strain (David Bergenholm, unpublished) where the native P_{OLE1} had been replaced with a glucose-regulated promoter, P_{HXT1}, which is reported to be down-regulated after glucose consumption [201]. Analysis of the fatty acid content of these strains shows that the modified strain produces much less unsaturated fatty acids (measure-

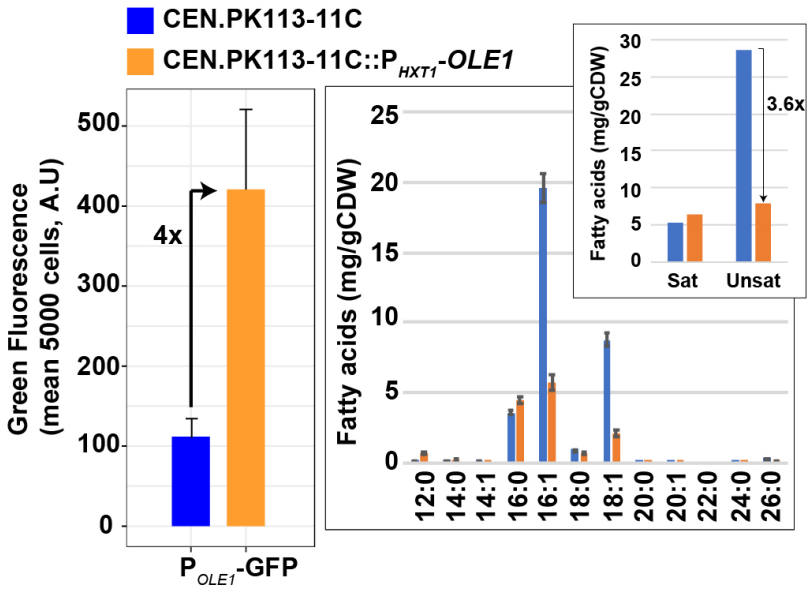

Figure 5.6 Saturated fatty acid-responsive biosensor. Saturated fatty acid-responsive sensor based on P_{OLE1} placed upstream of GFP on a centromeric plasmid. This sensor was expressed in both the wild-type strains and the strain having the endogenous P_{OLE1} replaced with P_{HXT1}, which has been shown to produce less unsaturated fatty acids. Sensor strains were analysed 24 h after inoculation using flow cytometry. The fatty acid levels were analysed from strains harvested 72 h after inoculation. n = 3, error bar = ± SD.

ments taken 72 h after inoculation). This change can also be observed using the sensor based on P_{OLE1}, which produces more fluorescence signal compared to the wild type-strain. P_{OLE1} is highly regulated by several factors [177], including changes in saturated/unsaturated fatty acid ratio where reduced levels of unsaturated fatty acids increases its activation (Figure 5.6).

As we and others have observed in previous studies, FadR works well as a biosensor in *S. cerevisiae*. However, expression of FabR resulted in a growth defect and gave inconclusive results when measuring the fluorescence signal. Generally, it is not desirable to have a sensor causing growth defect, especially if it is intended to be used for developing cell factories. In other words, a biosensor should ideally not compromise cell factory development [106]. Furthermore, expression of VcFadR and DesT did not result in reduced fluorescence, indicating that these are either strongly responsive to the fatty acid levels in the strain or that these do not function as repressors in *S. cerevisiae* (Figure 5.7). For *V. cholerae* FadR, the insufficient repression could be due to its higher affinity for fatty acids, as it has approximately 2.5-fold higher affinity towards oleoyl-CoA than *E. coli* FadR [200], which increases its sensitivity and thereby reduces its repression efficiency [110].

As FabR and DesT did not result in clear repression, we decided to evaluate how these transcription factors are being expressed in yeast. Therefore, we fused them to GFP using a flexible linker [202]. We observed that FabR did not seem to be well-expressed as indicated by

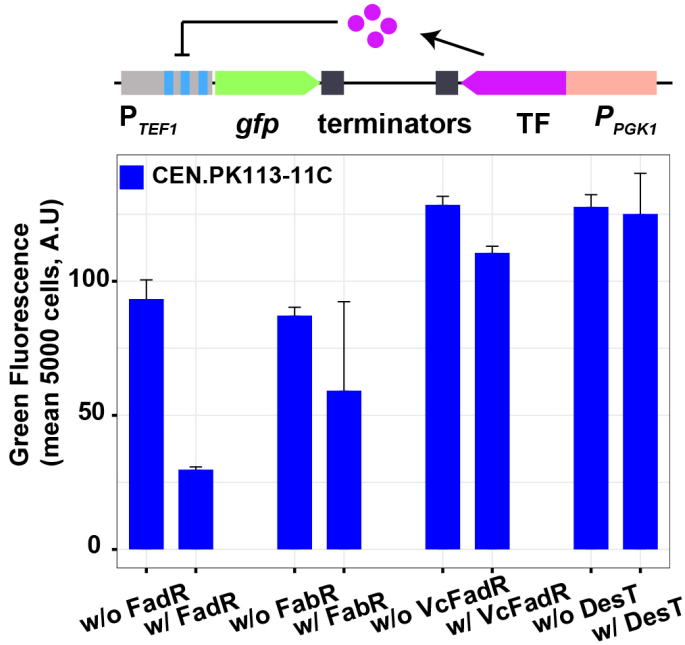

Figure 5.7 Long chain fatty acyl-CoA-responsive transcription factors. (A) Evaluation of different long-chain fatty acyl-CoA-responsive transcription factors; FadR (*E.coli*), FabR(*E. coli*), VcFadR (*V. cholerae*) and DesT (*P. aeruginosa*). The general setup was based on centromeric plasmids where 3 binding sites (blue strips) of each transcription factor were implemented in P_{TEF1}, which was placed upstream of the gene encoding GFP. The reporter promoters were expressed either without (w/o) or with (w) the transcription factors by placing the gene encoding each transcription factor downstream of P_{PGK1}. Samples were measured 8 h after inoculation using flow cytometry. n = 3, error bar = ± SD.

the small foci (Figure 5.8A) whereas DesT seemed to be well expressed and localized to the nucles (Figure 5.8B), although this was not confirmed through nucleus staining. In an earlier study [203], it was reported thar overexpression of FabR in its native host results in insoluble protein aggregates. Therefore, we placed FabR under promoters with weaker strength, such as P_{CYC1} instead of P_{PGK1}, but the growth was not improved (data not included). If what we observe in Figure 5.8A are aggregates, then one could potentially perform western blotting on the supernatant to more accurately analyse whether it is soluble or not. Recently, a more straightforward method was published [204] where a reporter system can be used to more readily evaluate protein aggregations in yeast, which could also be a useful approach to analyse expression of FabR. Another possibility is that what is seen in Figure 5.8A are protein degradations in endosomes [205]. Nonetheless, we decided to not continue further with these transcription factors, and looked instead inside (of *S. cerevisiae*) for another way to sense saturated fatty acids.

Expression of Ole1 has been reported to be tightly regulated by two transcription factors responsive to saturated fatty acids, namely Spt23 and Mga2 [206]. Although these are by definition not regarded as transcription factors (due to lack of DNA-binding domain), they

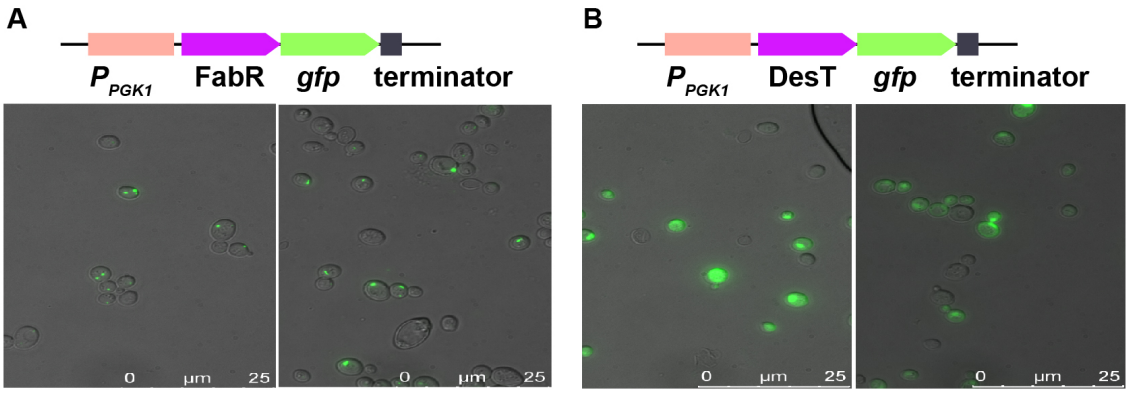

Figure 5.8 Microscopic evaluation of FabR and DesT fused with GFP. The genes encoding (A) FabR and (B) DesT were fused with GFP and placed downstream of P_{PGK1}. Microscopic evaluation of FabR-GFP fusion showed small foci, indicating that FabR potentially forms inclusion bodies whereas evaluation of DesT-GFP seemed to have a more expected GFP pattern. Samples were evaluated 6 h after inoculation.

have been reported to regulate transcription by modifying the chromatin structure, making it more accessible [207]. Although Mga2 and Spt23 have diverse regulatory functions, their main target is reported to be regulation of *OLE1* [207], in which Mga2 has a more dominant role [177, 208, 209]. These transcription factors are tethered to the ER membrane as 120-kDa, and processed to their 90-kDa activated form through a ubiquitin-mediated proteolysis system (Figure 5.9A). Upon increased saturated fatty acid levels, the 90 kDa domain is released and localized to the nucleus where it regulates *OLE1* expression [210].

We were interested in developing a saturated fatty acid-responsive biosensor based on Mga2 by employing a split GFP approach (Figure 5.9B) [211, 212]. By fusing one part of the GFP, GFP11, to Mga2 and the second part, GFP1-10, to an NLS tag, we expected to observed a GFP signal only when Mga2 is transported to the nucleus as the two GFP parts would theoretically re-assemble and thereby result in a functional protein. The intact version of GFP gave a strong fluorescent signal, even stronger than the signal observed from our standard GFP we use in the lab (Figure 5.10). Furthermore, expressing each part separately in a split-mode did also not result in a signal, which was a control to ensure that the parts do not give rise to fluorescence. However, when expressing the two parts together localized to the nucleus, we could not observe any GFP signal and neither could we observe a signal when expressing GFP11-Mga2 together with GFP1-10 (Figure 5.10).

In hindsight, the control we used, expressing GFP11 and GFP1-10 together, to evaluate the function of the split-GFP might not have been very reliable. The reason is that GFP11 consists of only 16 amino acid, and expressing it by itself might not have been very stable. Therefore, it might have been more reliable to evaluate the split system by tagging GFP11 to a protein we know is localized to the nucleus, for example FadR. Due to limited time, we decided here as well to set this project aside.

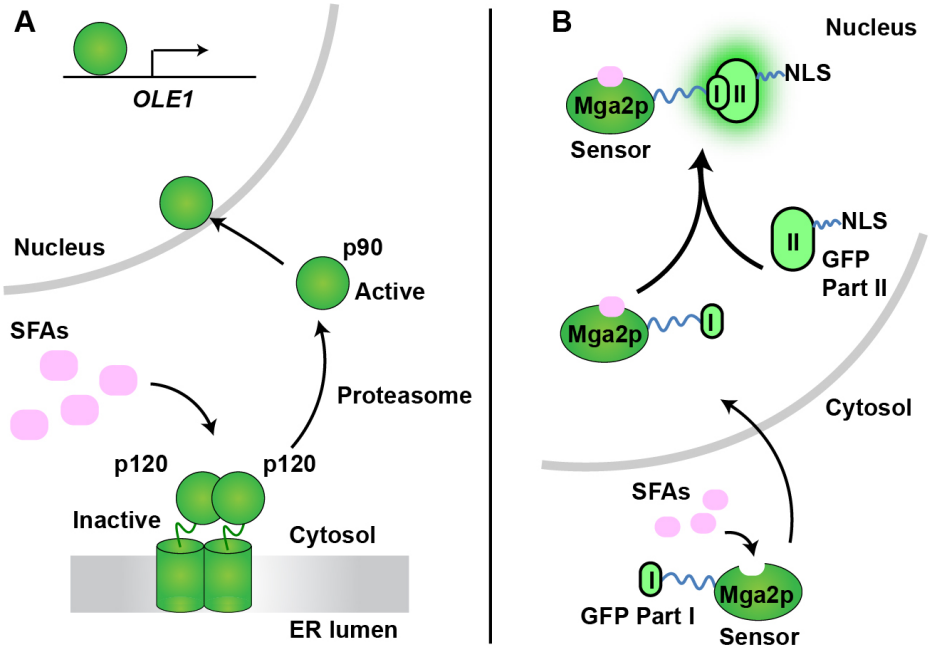

Figure 5.9 Design of a Mga2-based biosensor. (A) Mga2 has been reported to be an ER membrane-bound protein responsive to saturated fatty acids. In the presence of saturated fatty acids, Mga2 is ubiquitinilated to its smaller, active, form p90, which is transferred to the nucleus where it regulates *OLE1* expression. Figure adapted from [210]. (B) Design of a Mga2-based biosensor in *S. cerevisiae* using a split GFP approach.

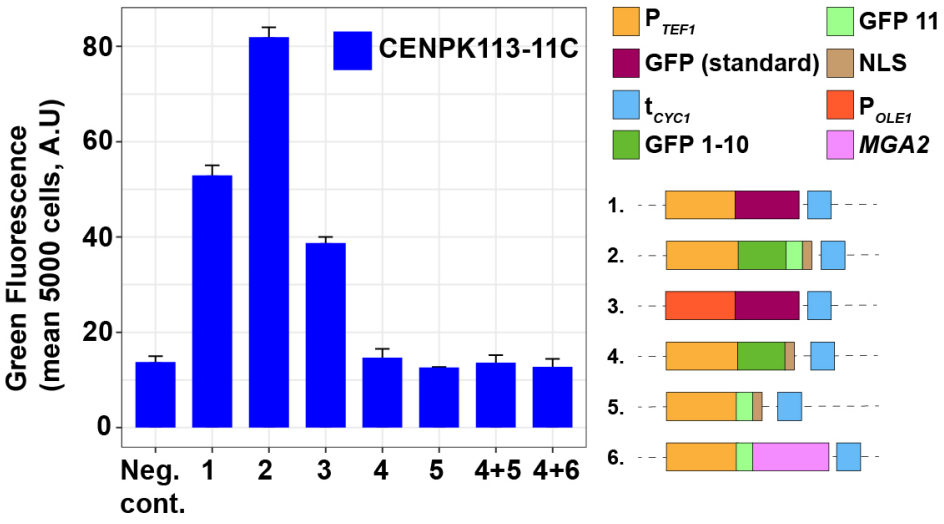

Figure 5.10 Evaluation of the Mga2-based biosensor. Different strains were evaluated by measuring fluorescence to determine the functionality of the Mga2-based biosensor. Samples were measured 8 h after inoculation using flow cytometry. n = 3, error bar = ± SD.

6 | Alkane-responsive biosensor

Alkanes, an important class of hydrocarbons, are additional industrially relevant compounds with the potential to be used for production of biofuels, specifically aviation fuels, with desirable properties such as high-energy density and low freezing points [213, 214]. There are microorganisms naturally producing alkanes [215], but the production levels and structure of the compounds are not ideal for commercial applications. Therefore, there has been a large body of research dedicated to engineer microorganisms including, *E. coli* and *S. cerevisiae*, to produce alkanes with improved, yet still low, titers [213].

There are several challenges for why it is difficult to produce commercially satisfying levels of alkanes [213]. One bottleneck is the low enzyme activity of an aldehyde-deformylating oxygenase, which converts aldehydes to alkanes (Figure 6.1). Therefore, one solution to improve alkane production is, for example, to employ directed evolution on this enzyme and sceen for strains having improved alkane production. To achieve this, an alkane-responsive biosensor would greatly facilitate such screening. In fact, an alkane-responsive biosensor based on prokaryotic activators has previously been developed in *E. coli* for screening of microbial cell factories [216]. However, since this is based on prokaryotic activators, and it is generally more complicated to implement prokaryotic activators successfully in eukaryotes due to the differences in the transcriptional machinery, we sought elsewhere for inspiration to develop an alkane-responsive biosensor in *S. cerevisiae*.

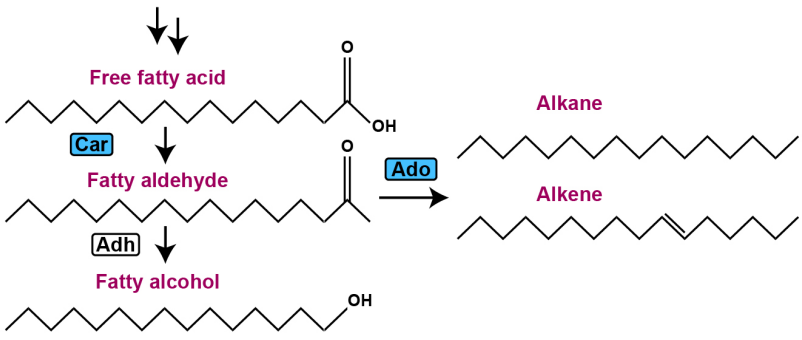

Figure 6.1 Heterologous alkane biosynthesis in *S. cerevisiae*. A potential route for alkane synthesis is through fatty aldehydes using the heterologous carboxylic acid reductase (CAR) enzyme and the aldehyde-deformylating oxygenase (ADO) enzyme to convert fatty aldehdyes to alka(e)nes.

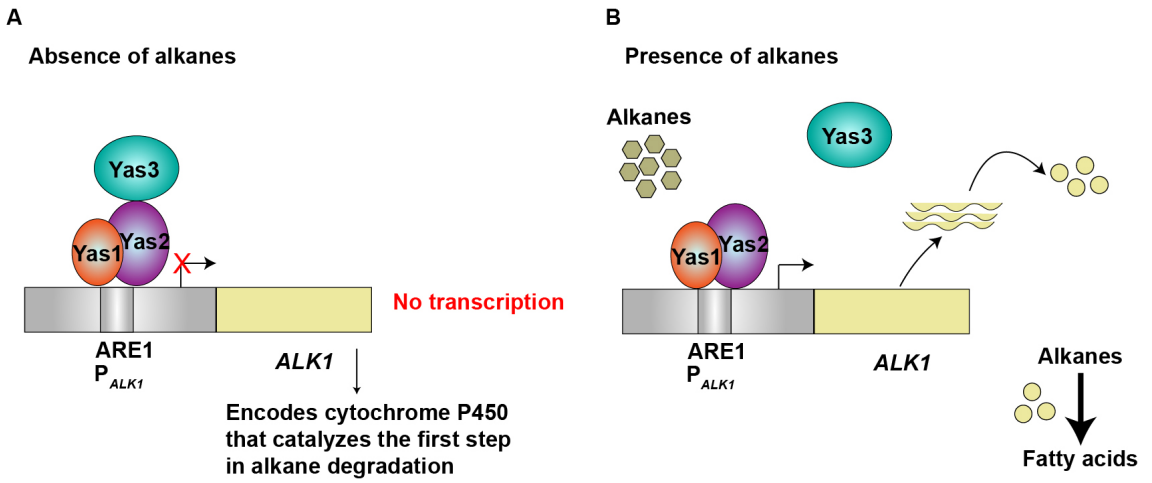

Figure 6.2 Putative alkane-sensing system in *Yarrowia lipolytica*. A) Yas1 and Yas2 have been reported to function as an activator complex, activating expression of the *ALK1* gene encoding a cytochrome P450 enzyme that is involved in catalyzing the first step in alkane degradation whereas Yas3 acts as a repressor upon binding to Yas2. This repression occurs in the absence of alkanes. B) On the other hand, activation occurs in the presence of alkanes, which results in re-localization of Yas3 to the endoplasmic reticulum. Adapted from [223].

6.1 Alkane-sensing system in *Yarrowia lipolytica*

Yarrowia lipolytica is an alkane-assimilating yeast, which lives in hydrophobic habitants and is able to utilize hydrophobic compounds, including alkanes [217]. There are several genes in *Y. lipolytica* encoding cytochrome P450ALKs, with *ALK1* being the most inducible in presence of alkanes [218]. It has been reported that Alk1, in the absence of alkanes, is negatively regulated by Yas3 binding to the heterodimeric activator complex Yas1 and Yas2 (Figure 6.2A) [219–221]. In the presence of alkanes, Yas3 has been reported to be re-localized to the endoplasmic reticulum, resulting in *ALK1* expression by Yas1-Yas2 (Figure 6.2B) [221]. In fact, Yas3 has been reported to be involved in regulating other genes, including a subset of other *ALK* genes in *Y. lipolytica*. However, an immediate challenge with this system is that there are many unknown mechanism that are yet to be elucidated [221, 222]. For example, the mechanism of how Yas3 is re-localized to the endoplasmic reticulum is still not fully understood.

6.2 Alkane-responsive biosensor

Despite the challenge of not having the alkane-sensing system fully elucidated, we still sought to develop an alkane-responsive biosensor based on the putative system in *Yarrowia lipolytica* (**Paper V**). As discussed previously, the common approach to develop transcription factor-based biosensors is to implement the corresponding binding sites into a well-characterized native yeast promoter. Here, we decided to implement the ARE1 binding site, to which

Yas1-Yas2 binds, in P_{CYC1} according to modifications done in a previous study [91] where the *CYC1* promoter was use for developing transcription factor-based biosensors based on prokaryotic activators in *S. cerevisiae*. We reasoned, therefore, that this set up would be suitable to use for observing activation upon expression of Yas1 and Yas2, and repression when expressing these together with Yas3. Since *Y. lipolytica* is also a yeast species, we decided to test whether its endogenous promoter P_{ALK1} would be functional in *S. cerevisiae*. Therefore, we decided to evaluate the alkane-sensing system consisting of Yas1, Yas2 and Yas3 using both the P_{ALK1} and P_{CYC1} promoters, from now on referred to as the P_{ALK1}-based system and P_{CYC1}-based system, respectively.

6.2.1 The P_{ALK1}- and P_{CYC1}-based systems

From the P_{ALK1}-based system, we observed several interesting results, including the increased GFP signal when expressing all three genes encoding the transcription factors Yas1, Yas2 and Yas3 together with P_{ALK1}, and a growth defect when expressing either Yas3 alone or together with Yas1 and Yas2 (Figure 6.3). To rule out the possibility that the observed GFP signal was due to autofluorescence from dead cells, we expressed all three genes encoding the transcription factors in another strain without the GFP protein. This did not result in any GFP signal, and together with the results observed from the propidium iodide staining (Figure 6.4), we could rule out the presence of GFP signal as a result of autofluorescence. It is, however, not clear why we observed increased GFP signal when combining all three transcription factors as this was expected to repress the system. Furthermore, when only expressing the genes encoding Yas1 and Yas2, activation could be observed when using flow cytometry, although this could not be observed during microscopic evaluation (**Paper V**).

In parallel to the P_{ALK1}-based system, we also evaluated the system based on P_{CYC1} (Figure 6.5). When expressing all three transcription factors together with modified versions of P_{CYC1}, we obtained inconclusive results due to high error bars. However, with the activator complex Yas1-Yas2, we observed an activation pattern, which surprisingly, was also observed for the unmodified *CYC1* promoter (Figure 6.5). After closer examination of the *CYC1* promoter, it turned out that a part of the motif, CTTGTGNX**CATGTG**, to which Yas1-Yas2 have been reported to bind to, also exists in the native *CYC1* promoter. Therefore, there is a possibility that Yas1-Yas2 activated the unmodified *CYC1* promoter due to similarities in binding site sequences [70]. In another study in *E. coli*, the repressor FapR was shown to have activating function upon interacting with a native *E. coli* promoter despite absence of *fapO* binding sites [90].

To understand better how these transcription factors are expressed, we fused each with GFP (Figure 6.5). Of all three transcription factors, Yas1 seemed to be the one properly expressed with an indicative expression in the nucleus (Figure 6.6B). However, no clear signal could be observed from Yas2 whereas a strong, but scattered, GFP signal was observed for Yas3 (Figure 6.6C-D). The scattered GFP signal observed for Yas3 might not be too

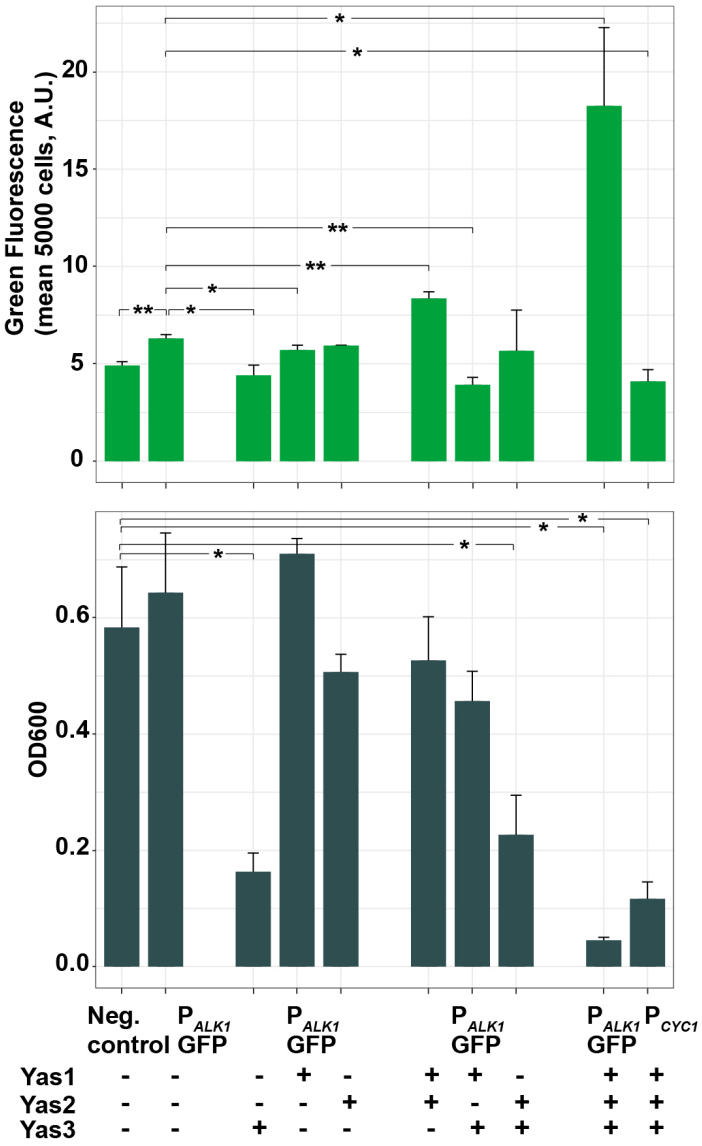

Figure 6.3 P_{ALK1}-based system. Fluorescence and OD600 measurements of strains carrying P_{ALK1}-GFP in combinations with the transcription factors Yas1, Yas2 and Yas3. The genes encoding the transcription factors were placed under the promoters P_{PGK1} (*YAS1* and *YAS2*) and P_{TEF1} (*YAS3*). Fluorescence and OD measurements were measured 6 h after inoculation using flow cytometry. n = 3, error bar = ± SD. **p*-value <0.05, ***p*-value <0.01 (Student's t test). Adapted from [223].

unexpected considering the growth defect it causes. Regarding Yas2, it has been reported that proteins functioning in complex with another transcription factor might be unstable without its counterpart [93, 224], potentially explaining the observed, weakened, GFP signal or the absence of it. However, there are several challenges observed when working with this system in *S. cerevisiae*, preventing its maturation into an alkane-responsive biosensor in its current form.

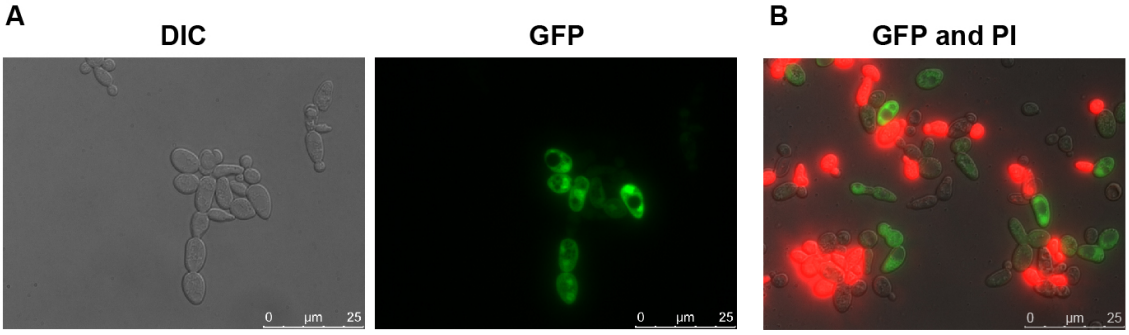

Figure 6.4 Microscopic evaluation of the P_{ALK1}-based system. A) Microscopic and fluorescence evaluation of strains carrying P_{ALK1} together with all three transcription factors Yas1, Yas2 and Yas3. B) Propidium iodide staining (PI) was used to evaluate whether the observed GFP signal was due to autofluorescence from dead cells. Samples were evaluated 28 h after inoculation. Adapted from [223].

6.2.2 Potential challenges of the *Y. lipolytica* alkane-sensing system

A system in *S. cerevisiae* resembling the Yas1-Yas2 and Yas3 system, is the heterodimeric Ino2-Ino4 activator and the transcription factor Opi1, which are global regulators regulating genes in the lipid biosynthesis but also genes unrelated to phospholipid metabolism [225]. Similar to Yas1-Yas2, Ino2-Ino4 contain a basic helix-loop-helix structure and Ino2 like Yas2 contains a trans-activating domain. An important motif (5'-CATGTGAAAT-3') bound by the Ino2-Ino4 complex is the inositol-responsive upstream activating sequence UAS_{INO}. Part of the motif in the ARE binding site, CTTGTGNX**CATGTG**, is found in the UAS_{INO}. In fact, the CANNTG motif is known to bind transcription factors, specifically heterodimers, with the basic helix-loop-helix motif [226]. This raises the potential that the activation observed for the unmodified *CYC1* promoter in the presence of Yas1-Yas2 may be due to similarities in the motifs, resulting in activation by Yas1-Yas2 irrespective of any ARE1 binding site in *CYC1*. In fact, Ino2-Ino4 has been shown to bind to P_{CYC1}. As it is beyond the scope of this thesis to go into the complex details of the Ino2-Ino4 and Opi1 system, I will here give a brief and simplified glimpse of a part of the system with the aim to relate this to the system in *Y. lipolytica*.

The UAS_{INO} element is found on promoter regions of many genes involved in phospholipid- and fatty acid biosynthesis. UAS_{INO}-containing genes are regulated in response to the availability of phospholipid precursors, inositol and choline [227]. Since inositol and choline enter the pathway for phospholipid synthesis by different routes, they influence lipid metabolism regulation in distinctly different ways. For example, when inositol levels are scarce, Opi1 is retained in the ER, partly through interaction with phosphatidic acid, which is a precursor to phosphatidylinositol and other phospholipids. Therefore, in the absence of inositol, phosphatidic acid levels are elevated due to reduced phosphatidylinositol synthesis, which retains

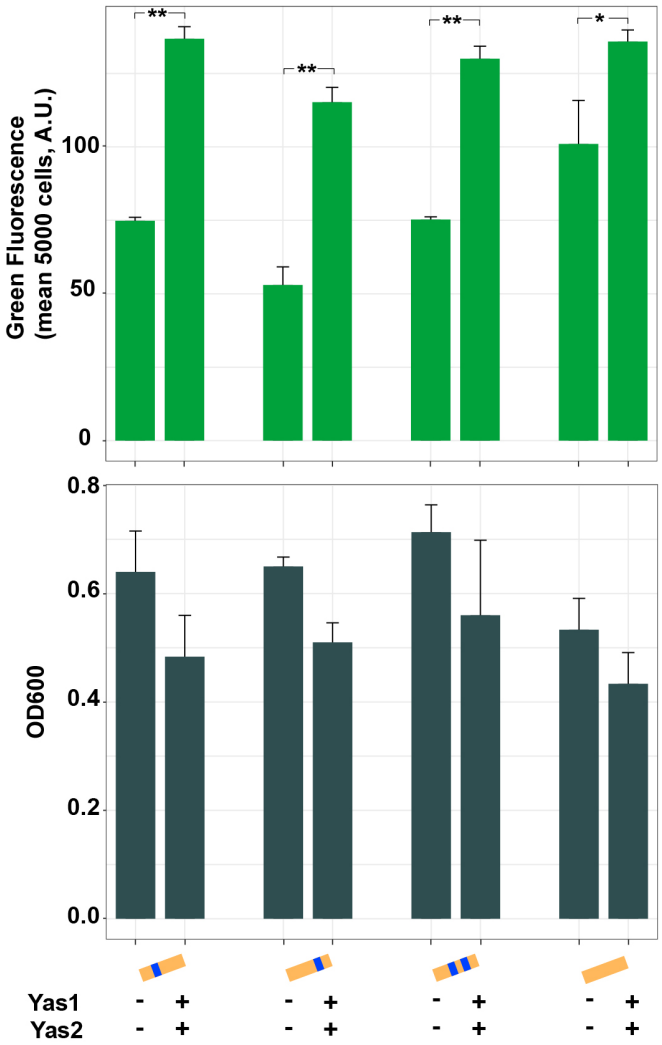

Figure 6.5 P_{CYC1}-based system. Fluorescence and OD600 measurements of strains carrying P_{CYC1}-GFP in combinations with the transcription factors Yas1 and Yas2. The genes encoding Yas1 and Yas2 were placed under the promoter P_{PGK1}. Fluorescence and OD600 were measured 6 h after inoculation using flow cytometry. n = 3, error bar = ± SD. *p-value <0.05, **p-value <0.01 (Student's t test). Adapted from [223].

Opi1 in the endoplasmic reticulum, resulting in activation of *INO1*, encoding *myo*-inositol-3-phosphate synthase, and other UAS_{INO}-containing genes by Ino2-Ino4. On the other hand, in presence of inositol, phosphatidic acid reduces, resulting in the re-localization of Opi1 to the nucleus where it interacts with Ino2 and represses the activating function. Although the system in *Y. lipolytica* has not been shown to respond to inositol [221], there are striking similarities in the structure of Yas3 and Opi1 and their differential localization, indicating that the system found in *Y. lipolytica* may be as complex as the system in *S. cerevisiae*. Due to many mechanism potentially yet to be elucidated, it is challenging to figure out the reasons for the observed behaviours of the Yas1-Yas2 and Yas3 in **Paper V**. For example, there is a

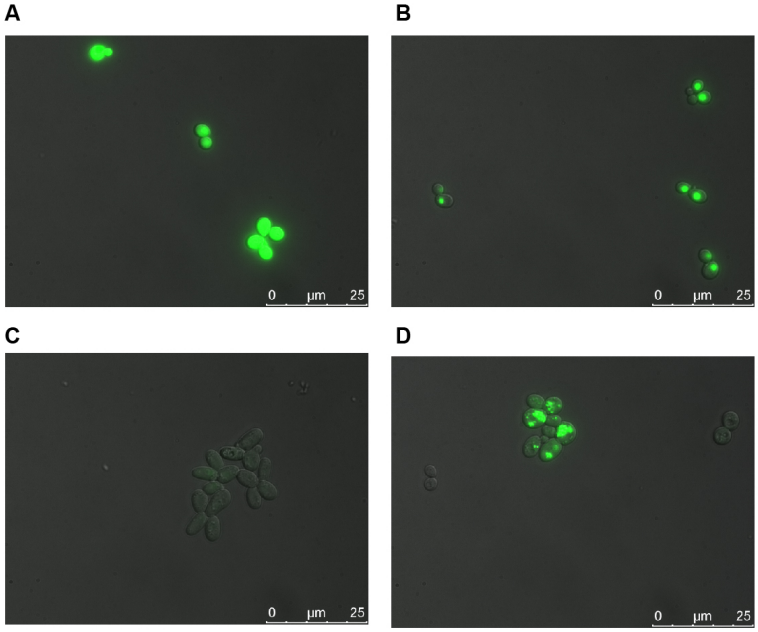

Figure 6.6 Evaluation of transcription factor-GFP fusion proteins. A) P_{TEF1}-GFP was used as a control, B) Yas1 fused to GFP under the control of P_{PGK1}, C) Yas2 fused to GFP under the control of P_{PGK1} and D) Yas3 fused to GFP under the control of P_{TEF1}. Samples were evaluated 6 h after inoculation. Taken from [223].

possibility that Yas3 interacts with several other components in *S. cerevisiae*, and to obtain a better understanding of such potential interactions one could perform a transcriptome analysis and evaluate whether certain genes are up- or downregulated in the presence of Yas3. Furthermore, a more precise analysis of the expression of the genes encoding the transcription factors would be through qRT-PCR and their production through western blotting.

When expressing the genes we used medium to strong promoters, including P_{PGK1} and P_{TEF1}. We did evaluate the system using the weaker promoter P_{REV1} for expressing *YAS3*, and although we observed reduced growth defect we still did not obtain clear results (data not shown) and decided therefore to not evaluate this further. In general, when developing a biosensor it is crucial that its components are well-characterized, especially if the aim is to engineer this further to make it more user-specific. However, this study is a clear demonstration of the challenge of developing a biosensor, especially based on a multi-component system, without sufficient knowledge about the mechanism of the transcription factors.

PART FOUR

Past and Future

7 | Conclusions and Outlook

The complexity of biological systems, their inherently robust and flexible networks and their ability to adapt to changing surroundings combined with our limited understanding, has made biological engineering one of the most challenging engineering disciplines. Despite this, biotechnology and many of its subfields are witnessing great expansions driven by its great potential to, for example, advance healthcare related endeavours and divert from petroleum-based production to more environmentally friendly alternatives.

Utilizing living systems, or cell factories, to produce many of our commodity chemicals and pharmaceuticals has proven successful in several cases. To increase the success rate, however, and move from laboratory-scale to industrial-scale fermentations, more sophisticated tools are necessary. It is generally agreed that one such tool are metabolite biosensors, allowing productive strains to be more readily and quickly identified as well as making production strains more robust through dynamic pathway regulation. Furthermore, a much greater diversity of cells can be evaluated, increasing the possibilty of finding a unique combination. Finally, the learning step would also accelerate as identifiying and analyzing unproductive strains can offer a great learning opportunity, allowing smarter designs and smarter libraries to be developed.

Conclusions: The work in this thesis has been focused on developing metabolite biosensors for accelerating cell factory development. In the early days of my PhD studies, it was clear that our main focus would specifically be on developing transcription factor-based biosensors, preferably for fatty acids. With the limited knowledge that I had, it all seemed very straightforward and clear. *How difficult could it actually be? We put the parts together and look for the signal we desire*. However, as it has probably been evident through this thesis, things were not always running smoothly. Sometimes we got what we expected just to realize after repeated measurements that we could not have been further away from the desired results, but definitely closer to the harsh reality reminding us that biology has its own way of being. Now, looking back on the work performed during the last 4 years, a valid question would be "*So what?*". Although some of the projects in this thesis did not result into papers, and although we lacked the time to dig deeper and properly investigate the causes, we do believe that these unfinished projects have scientific merit that might be worth looking into in the future. However, the main contribution of this thesis to the scientific community is probably

through the published papers.

In our review, **Paper I**, we provide a recent overview of the strategies and challenges for rewiring metabolism. In **Paper II**, we used the well-characterized bacterial transcription factor FapR to demonstrate the important role of operator site positioning and how the maximal dynamic range can be improved. Evaluating different reporter promoters not commonly used for biosensor development can increase the biosensor toolbox and contribute with better understanding of operator positioning. In **Paper III**, we used dCas9-VPR and transcription factor binding data of Gcr1, Gcr2 and Tye7 to investigate the effect of dCas9-VPR binding on top of identified motifs or outside of these in 10 glycolytic promoters. We observed a pattern suggesting that there is either a synergistic activation when dCas9-VPR is bound outside of identified motifs or a competition with respective transcription factor when dCas9-VPR is bound on top of the motifs. We further suggest that using this approach might help improve our understanding of promoter architechture and thereby facilitate biosensor development. In **Paper IV**, we employed a fatty acyl-CoA-reponsive biosensor based on the bacterial transcription factor FadR to screen for genes increasing the fatty acyl-CoA pool. This approach could be used to find promising gene candidates relevant for metabolic engineering. Finally, in **Paper V**, we sought to develop an alkane-based biosensor but encountered a number of challenges, suggesting that the system in its current form is not mature to be translated into an alkane-responsive biosensor. However, the possibility of developing an alkane-responsive biosensor based on this system has yet not been ruled out. In summary, this thesis has focused on metabolite-responsive transcription factor-based biosensors that are of scientific interest and industrial importance.

Outlook: Metabolite biosensors are attractive synthetic biology tools with applications in several engineering endeavours. Their development, however, is slow and often limited to a few well-characterized sensors. A challenge here is the need of more standardized parts and their fundamental understanding and applicability in a wide range of hosts and conditions. Without sufficient fundamental research, applied research can be incredibly time-consuming.

For transcription factor-based biosensors, the majority of transcription factors are derived from prokaryotes, and in addition to the challenge of finding suitable transcription factors, is the process of onboarding these into yeast. Since promoters are an integral part of transcription factor-based biosensors, their efficient integration is key. With detailed knowledge of promoter architechture and predictable gene expression outcomes, some of the challenges we encounter today would have been minimal or non-existent. Therefore, with better understanding of promoters, the importance and influence of sequences and the identification of regulatory elements and their interaction with transcription factors and other regulatory proteins could greatly facilitate promoter engineering endeavours. For example, employing machine learning approaches to study gene expression has allowed researchers to obtain a better understanding of the role DNA sequences play in such regulation [228]. Employing similar strategies in the future will definitely increase our understanding of factors influencing

gene regulation and thereby assisting in analysing and extracting biological information for biosensor development. Although engineering the reporter promoter can influence the response curve, including the dynamic- and operational range, there are other ways to facilitate biosensor development, such as engineering the transcription factor itself. For example, the specificity of transcription factors, which has not been discussed much in this thesis, is an important parameter that can render a transcription factor useful or useless for its purpose.

The specificity and other features of a biosensor can be modified or improved through various engineering strategies, for example through random mutagenesis or constructing large-scale libraries [66, 136]. However, this process would have been more efficient the better understanding we have of the sequence to function relationship. Therefore, we envision that collecting large-scale data-sets to support the development of machine-learning algorithms can immensely improve the predictiveness of future engineering and in general increasing our understanding of biosensor function [229]. Ultimately, this might result in more standardized biosensor engineering principles, which are currently lacking.

Ideally, biosensor engineering guidelines should be extended beyond a specific parameter and additionally include a model to predict or simulate the biosensor behaviour such as its dynamic- and operational range. This dedication is especially relevant for biosensors that have found application in a wide range of studies, such as FapR-based biosensor [67]. Since biosensors usually have to be engineered to fit a specific application, it would be great to be able to evaluate certain parameters *in silico* instead of the more laborious work *in vivo*.

Although high-throughput screening tools such as metabolite biosensors are promising for accelerating cell factory development, further improvements in standardization of biological parts and their characterization as components of integrated, complex systems is necessary in order to improve predictability with increased combinations. Furthermore, development of more predictive models through combination of big data and integrative analysis and the use of aritifical intelligence and machine learning will be needed to decrease the costs and increase the speed of the design-build-test-learn cycle. In addition, other persisting challenges exists that might hinder an efficient establishment of cell factories in our society, for which it is ultimately developed for. For example, the key driver for chemical industries should, ideally, not be to produce biologically derived chemicals only if these have better properties than traditional chemicals or chemicals that can find novel applications. The environmental aspect should play an important part, and the desire to reduce petroleum-driven chemicals should not only be encouraged by governments but should be highly prioritized. To move towards a circular economy, the society at large needs to be well-informed through joint forces and transparent communication between governments, industries, researchers and the public. Finally, although some countries have witnessed significant advancements towards a circular economy, there are still many countries living according to the 'take-make-waste' linear model, which prevents sustainability to be achieved on a global level. Despite the challenges, we must also acknowledge the incredible advancements achieved so far in the 21st century, where biotechnology has played a visible and significant role.

Appendix

In the following tables are listed transcription factor-based biosensor papers for (potential) metabolic engineering applications. It should be noted that these lists might not be complete and other relevant papers might have been missed out. Furthermore, the studies have been divided into three sections, including high-throughput screening/selection (HTS), dynamic pathway regulation (DPR) and demonstration (DEM), based on the focus of the study. Demonstrations, including proof-of-concept studies, is a broad section including papers based on biosensor development, real-time monitoring, modelling and biosensor engineering, including engineering the specificity and the response curve.

Table 7.1: Transcription factor-based biosensor papers published year 2020.

References	**Host**	**Use**
Wang et al. (https://doi.org/10.1021/acssynbio.9b00477)	*S. cerevisiae*	HTS
Ambri et al. (https://doi.org/10.1021/acssynbio.9b00333)	*S. cerevisiae*	DEM
Qiu et al. (https://doi.org/10.1186/s12934-020-01405-1)	*S. cerevisiae*	DEM
Wei et al. (https://doi.org/10.1021/acssynbio.0c00122)	*Y. lipolytica*	DEM
Wen et al. (https://doi.org/10.1021/acssynbio.9b00378)	*K. phaffii*	DPR
Qiu et al. (https://doi.org/10.1016/j.ymben.2020.03.006)	*E. coli*	HTS
D'Ambrosio et al. (https://doi.org/10.1016/j.biotno.2020.01.002)	*S. cerevisiae*	DEM
Snoek, T., et al. (https://doi.org/10.1093/nar/gkz954)	*S. cerevisiae*	DEM
Berepiki, A., et al. (https://doi.org/10.1021/acssynbio.9b00448)	*E. coli*	DEM
Corte, D., et al. (https://doi.org/10.1038/s41467-020-18400-0)	*C. glutamicum*	DEM
Zhang, J., et al. (https://doi.org/10.1038/s41467-020-17910-1)	*S. cerevisiae*	DEM
D'Ambrosio et al. (https://doi.org/10.1016/j.ymben.2020.07.006)	*S. cerevisiae*	DPR
Hanko, E., et al. (https://doi.org/10.1038/s41467-020-14941-6)	*C. necator*	DEM
Ding, N., et al. (https://doi.org/10.1093/nar/gkaa786)	*E. coli*	DEM

Table 7.2: Transcription factor-based biosensor papers published year 2019.

References	Host	Use
Yu et al. (https://doi.org/10.1186/s12934-019-1084-2)	*E. coli*	HTS
Dabirian et al. (https://doi.org/10.1021/acssynbio.9b00118)	*S. cerevisiae*	HTS
Dabirian et al. (https://doi.org/10.1021/acssynbio.9b00144)	*S. cerevisiae*	DEM
Nguyen et al. (https://doi.org/10.1007/s12257-018-0380-8)	*P. denitrificans*	DEM
Kortmann, M., et al. (https://doi.org/10.1021/acssynbio.8b00510)	*C. glutamicum*	DEM
Thompson, M., et al. (https://doi.org/10.1021/acssynbio.9b00255)	*P. putida*	DEM
Machado, L., et al. (https://doi.org/10.1186/s13036-019-0214-z)	*E. coli*	DEM
Flachbart, L., et al. (https://doi.org/10.1021/acssynbio.9b00149)	*E. coli*	HTS
Monteiro, F., et al. (https://doi.org/10.15252/msb.20199071)	*S. cerevisiae*	DEM

Table 7.3: Transcription factor-based biosensor papers published year 2018.

References	Host	Use
Zhao, E., et al. (https://doi.org/10.1038/nature26141)	*S. cerevisiae*	DPR
Liu, C., et al. (https://doi.org/10.1021/acssynbio.7b00339)	*C. glutamicum*	DEM
Kim, S., et al. (https://doi.org/10.1021/acssynbio.8b00164)	*E. coli*	DEM
Seok, J., et al. (https://doi.org/10.1016/j.ymben.2018.03.009)	*E. coli*	HTS
Zheng, S., et al. (https://doi.org/10.1016/j.ymben.2018.08.005)	*E. coli*	HTS
Rebets, Y., et al. (https://doi.org/10.1016/j.ymben.2018.03.019)	*S. alboniger*	DEM
Woolston, B., et al. (https://doi.org/10.1002/bit.26455)	*E. coli*	DEM
Hanko, E., et al. (https://doi.org/10.1021/acssynbio.8b00057)	*E. coli*	DEM
Frazao, C., et al. (https://doi.org/10.3389/fbioe.2018.00118)	*E. coli*	DEM
Chen, X., et al. (https://doi.org/10.3389/fmicb.2018.00047)	*S. cerevisiae*	DEM
Baumann, L., et al. (https://doi.org/10.1021/acssynbio.8b00309)	*S. cerevisiae*	DEM
Doong, S., et al. (https://doi.org/10.1073/pnas.1716920115)	*E. coli*	DPR
Snoek, T., et al. (https://doi.org/10.1021/acssynbio.7b00439)	*S. cerevisiae*	HTS
De Paepe, B., et al. (https://doi.org/10.1021/acssynbio.7b00419)	*E coli*	DEM
Juarez, J., et al. (https://doi.org/10.1038/s41467-018-05525-6)	*E coli*	DEM
Trabelsi, H., et al. (https://doi.org/10.1002/bit.26726)	*E coli*	POC
Peters, G., et al. (https://doi.org/10.1002/bit.26586)	*E coli*	DEM
Jester, B., et al. (https://doi.org/10.1021/acssynbio.8b00242)	*S. cerevisiae*	DEM
Younger, A., et al. (https://doi.org/10.1093/protein/gzy001)	*E. coli*	DEM

Table 7.4: Transcription factor-based biosensor papers published year 2017.

References	Host	Use
Hector, R., and Mertens, J. (https://doi.org/10.1007/s12033-016-9991-5)	*S. cerevisiae*	DEM
Xiong, D., et al. (https://doi.org/10.1016/j.ymben.2017.01.006)	*E. coli*	HTS
Chen, X-F., et al. (https://doi.org/10.1002/bit.26521)	*E. coli*	DEM
Leavitt, J., et al. (https://doi.org/10.1002/biot.201600687)	*S. cerevisiae*	HTS
Liu, Y., et al. (https://doi.org/10.1021/acssynbio.6b00328)	*E. coli*	HTS
Li, H., et al. (https://doi.org/10.1016/j.bios.2017.07.022)	*E. coli*	HTS
Kasey, C., et al. (https://doi.org/10.1021/acssynbio.7b00287)	*E. coli*	HTS
Siedler, S., et al. (https://doi.org/10.1021/acssynbio.7b00009)	*E. coli*	HTS
Williams, T., et al. (https://doi.org/10.1093/synbio/ysw002)	*S. cerevisiae*	HTS
Mannan, A., et al. (https://doi.org/10.1021/acssynbio.7b00172)	*E. coli*	DEM
Wu, J., et al. (https://doi.org/10.1038/srep45994)	*E. coli*	HTS
Li, H., et al. (https://doi.org/10.1186/s12934-017-0794-6)	*E. coli*	HTS
Liang, W-F., et al. (https://doi.org/10.1016/j.ymben.2016.11.010)	*M. extorquens*	HTS
Hanko, E., et al. (https://doi.org/10.1038/s41598-017-01850-w)	*C. necator*	DEM

Table 7.5: Transcription factor-based biosensor papers published year 2016.

References	Host	Use
David, F., et al. (https://doi.org/10.1021/acssynbio.5b00161)	*S. cerevisiae*	DPR
Leavitt, J., et al. (https://doi.org/10.1002/biot.201600029)	*S. cerevisiae*	DEM
Taylor, N., et al. (https://doi.org/10.1038/nmeth.3696)	*E. coli*	DEM
Rogers, J., and Church, G. (https://doi.org/10.1073/pnas.1600375113)	*E. coli*	DEM
Skjoedt, M., et al. (https://doi.org/10.1038/nchembio.2177)	*S. cerevisiae*	DEM
Zhang, J., et al. (https://doi.org/10.1021/acssynbio.6b00135)	*S. cerevisiae*	DEM
Machado, L., et al. (https://doi.org/10.1039/C6CC04559f)	*E. coli*	DEM
Libis, V., et al. (https://doi.org/10.1021/acssynbio.5b00225)	*E. coli*	DEM
Younger, A., et al. (https://doi.org/10.1021/acssynbio.6b00184)	*E. coli*	DEM
Xiao, Y., et al. (https://doi.org/10.1038/nchembio.2046)	*E. coli*	DEM

Table 7.6: Transcription factor-based biosensor papers published year 2015.

References	Host	Use
Li, S., et al. (https://doi.org/10.1021/acssynbio.5b00069)	*S. cerevisiae*	HTS
Wang, M., et al. (https://doi.org/10.1002/bit.25676)	*S. cerevisiae*	HTS
Feng, J., et al. (https://doi.org/10.7554/eLife.10606.001)	*S. cerevisiae*	DEM
Teo, W., and Chang, M. (https://doi.org/10.1002/biot.201400159)	*S. cerevisiae*	DEM
Chen, W., et al. (https://doi.org/10.1016/j.ymben.2015.05.004)	*E. coli*	HTS
Mahr, R., et al. (https://doi.org/10.1016/j.ymben.2015.09.017)	*C. glutamicum*	HTS
de los Santos, E., et al. (https://doi.org/10.1021/acssynbio.5b00090)	*E. coli*	DEM
Rogers, J., et al. (https://doi.org/10.1093/nar/gkv616)	*E. coli*	DEM
Wu, W., et al. (https://doi.org/10.1038/srep10907)	*E. coli*	DEM
Feher, T., et al. (https://doi.org/10.3389/fbioe.2015.00046)	*E. coli*	DEM
Urgel-Espinosa, M., et al. (https://doi.org/10.1007/s12033-015-9849-2)	*P. putida*	DEM

Table 7.7: Transcription factor-based biosensor papers published year 2014.

References	Host	Use
Xu, P., et al. (https://doi.org/10.1073/pnas.1406401111)	*E. coli*	DPR
Teo, W., and Chang, M. (https://doi.org/10.1002/bit.25001)	*S. cerevisiae*	DEM
Xu, P., et al. (https://doi.org/10.1021/cb400623m)	*E. coli*	DPR
Xue, H., et al. (https://doi.org/10.1021/sb500023f)	*E. coli*	DEM
Siedler, S., et al. (https://doi.org/10.1021/sb400110j)	*E. coli*	DEM
Siedler, S., et al. (https://doi.org/10.1016/j.ymben.2013.10.011)	*E. coli*	HTS
Mustafi, N., et al. (https://doi.org/10.1371/journal.pone.0085731)	*C. glutamicum*	DEM
Jha, R., et al. (https://doi.org/10.1093/nar/gku444)	*E. coli*	HTS
Schendzielorz, G., et al. (https://doi.org/10.1021/sb400059y)	*C. glutamicum*	HTS
Raman, S., et al. (https://doi.org/10.1073/pnas.1409523111)	*E. coli*	HTS

Table 7.8: Transcription factor-based biosensor papers published year 2013.

References	Host	Use
Teo, W., et al. (https://doi.org/10.1002/elsc.201200113)	*S. cerevisiae*	DEM
Liu, D., et al. (https://doi.org/10.1021/sb400158w)	*E. coli*	DPR
Dietrich, J., et al. (https://doi.org/10.1021/sb300091d)	*E. coli*	HTS
Tang, S-Y., et al. (https://doi.org/10.1021/ja402654z)	*E. coli*	HTS
Choi, H., and Keasling, J. (https://doi.org/10.1038/ncomms3595)	*E. coli*	HTS
Umeyama, T., et al. (https://doi.org/10.1021/sb300115n)	*S. cerevisiae*	HTS
Choi, S-L., et al. (https://doi.org/10.1021/sb400112u)	*E. coli*	HTS

Table 7.9: Transcription factor-based biosensor papers published year 2012.

References	Host	Use
Reed, B., et al. (https://doi.org/10.1016/j.jbiotec.2012.01.028)	*E. coli*	HTS
Binder. S., et al. (https://doi.org/10.1186/gb-2012-13-5-r40)	*C. glutamicum*	HTS
Mustafi, N., et al. (https://doi.org/10.1016/j.ymben.2012.02.002)	*C. glutamicum*	HTS
Zhang, F., et al. (https://doi.org/10.1038/nbt.2149)	*E. coli*	DPR

Table 7.10: Transcription factor-based biosensor papers published year 2000-2011.

References	Host	Use
Tang, S-Y., and Cirino, P. (https://doi.org/10.1002/anie.201006083)	*E. coli*	HTS
Uchiyama, T., and Miyazaki, K. (https://doi.org/10.1128/AEM.00464-10)	*E. coli*	HTS
Tang, S-Y., et al. (https://doi.org/10.1021/ja7109053)	*E. coli*	HTS
van Sint Fiet, S., et al. (https://doi.org/10.1073/pnas.0504733102)	*E. coli*	HTS
Lee, S., and Keasling, J. (https://doi.org/10.1002/bit.20784)	*E. coli*	DEM
Baker, K., et al. (https://doi.org/10.1073/pnas.262420099)	*S. cerevisiae*	HTS
Farmer, W.R., and Liao, J.C. (https://doi.org/10.1038/75398)	*E. coli*	DPR

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