Distributed computation in multicellular synthetic networks

Arturo Urrios Garcia

PhD Thesis UPF

Barcelona, 2017

Treball dirigit pel Dr. Francesc Posas Garriga i la Dra. Eulàlia de Nadal Clanchet

Departament de Ciències Experimentals i de la Salut (DCEXS)

Programa de Doctorat en Biomedicina de la Universitat Pompeu Fabra

Be yourself, everyone else is already taken

–OSCAR WILDE

ACKNOWLEDGMENTS

In Spain, we say that there are three things everyone should do before dying: have a kid, plant a tree and write a book. At the moment, having a kid is not on my daily agenda and I've tried my luck with plants and so far, I've failed. So, at least, I hope that writing the thesis will count as writing a book.

Writing the thesis is the easiest part of the journey, you just sit in front of your computer, sum up your results and start thinking how did you get here. Soon, you realize that many things have happened and many people have been there in your good and bad moments, and the moment you grasp that, you also realize that there's a lot to be thankful for.

First of all, I thank Dr. Francesc Posas and Dra. Eulàlia de Nadal, my thesis supervisors, for giving me the opportunity to join their group where I met my second family. Francesc, Laia, you gave me the opportunity to start my thesis at your lab. During these years you have given me projects and provide guidance but at the same time you have given me freedom to explore my own ideas. For all this, you have my deepest thanks. Also, I want to thank Obra Social "La Caixa" who trusted me and gave me a fellowship and Centre per a la Innovació de la Diabetis Infantil Sant Joan de Déu (CIDI) who partially funded the work presented here, without their support things would have not been the same.

In the Cell Signaling Research Group I've met a lot of interesting people that have helped me, taught me and cheered me up every single day, you know what they say, friendship can end but lab partners are forever. I want to thank all of them, specially: the mammals (Manel, Arnau, Ramon, Pedro, Cate, Sina, Predrag and Maria), the cell cycle unit (Alba, Tognetti, Berta, Javi and Alberto, the first ones to teach me in the lab), the transcription unit (Carme, Mariona, Jorge, Santi, Gerard, Du, Cris, Martina, Marc and

Andrea), the cell comput unit (Sergi, David, who although he was the last one to arrive he merged in as if he was a part of the team from the beginning, Javier and Eva, who started knowing nothing and now is pursuing her doctoral degree), the targets unit (Seisi, Jana and Anna, who shared our space turning our hybrid unit into the most cheerful unit of the lab) and the other members of the lab: Montse and Laia, whose friendship will last for ages; Aida, who has always cared to make sure that everything was ready to use, and the bioinformaticians Pablo and Rene, who also encouraged me on pursuing my “business ideas”. Thank you all for sharing your time with me, friends.

I also want to thank the former and current members from virology (Marc, Leire, Bernat. Jenni and Gemma); the members from immunology, specially Laia and Hector, we have shared a lot of stories playing beach volleyball and having fun; the former and current members from Pombe, specially Isa, who was the first person to teach me how to work in a lab, Esther, Sarela, Merce, Marga, Montse, Rodrigo, Alberto, Luis, Cristina, Esther, Claudia and Alba, my sister-in-arms who is currently polishing her thesis manuscript, we have shared so many funny moments, and also I want to thank their PIs, Dr. José Ayté and Dra. Elena Hidalgo, who were the first ones to introduce me in the world of academic science, thanks for trusting me while I was still an undergraduate student.

I also want to thank Dr. Albert Folch and the members of the Folch lab in Seattle (Lisa, Alan, Luis, Cesar and Nirveek), where I spent 9 months. Thanks Albert for introducing me into the world of 3D printing and microfluidics and trusting me, even though I was a biologist with no prior real experience on the field of engineering.

You are a role model. Also, I want to thank other people that I've met in the US and changed me (Lonnie, Collenn and Claudia).

I also want to thank my best friends who have been there since I was a kid (Dani, Iván and Carlos) and my classmates (who are more than classmates) from the MBA for their support and "fun moments" (Albert, Albert, Alfredo, Angel, Carles, Fatima, Javier, Jordi, Jordi, Jorge, Judit, Karim, Luli, Marina, May, Merce, Miquel, Mireia, Nico, Oriol, Rodri and Tamara).

And now allow me to change to Spanish to thank my family, so they can fully understand the meaning of my words.

A mi familia quiero agradeceros vuestro apoyo, los ánimos y las experiencias que me habéis bridando estos 26 años que llevamos juntos. Es difícil de imaginar que sentís, porque habéis estado a mi lado incluso antes de que yo fuera consciente de que existíais. En estos años, me habéis visto crecer, dar más de dos pasos y conseguir decir mis primeras palabras; me habéis visto crecer y desarrollarme como persona; me habéis visto crecer y entrar en la vida adulta; me habéis visto crecer y elegir mi vocación; me habéis visto crecer a vuestro lado y a través de la pantalla de un ordenador en la otra punta del mundo, pero siempre me habéis visto crecer y seguir adelante, y aunque una tesis pueda parecer el fin de una etapa, seguiré creciendo y seguiréis estando allí para verlo. Realmente, las palabras se quedan cortas para expresar mi gratitud, pero quiero agradeceros vuestro apoyo, a mi padre, madre, hermano, abuelos, abuelas, tíos, tías, primos y primas, que siempre han estado allí.

Arturo Urrios Garcia
07 September 2017
Somewhere in Russia

SUMMARY

Biological systems gather information from the environment and perform computations at multiple levels. Advancements in molecular biology and techniques for cell manipulation, genome engineering and DNA synthesis among others, allowed to rationally engineer biological systems, giving rise to a new biological discipline called synthetic biology (Manzoni, Urrios et al., 2016).

Currently the field is focused mainly on implementing gene expression based devices in single cells. However, this approach may have some scalability problems, such as the wiring problem. To overcome this limitation, we proposed the use of multicellular networks to build circuits (Regot et al., 2011).

In this PhD thesis, we have studied the use of multicellular circuits to perform biological computations in different scenarios:

On one hand, we have addressed the scalability problem by coupling multicellular circuits using *S.cerevisiae* with spatial segregation. Our goal was to build complex circuits with the minimal cell engineering possible. Our results showed that minimizing circuits to one-input logic gates, connecting cells by a single wire and placing them in different chambers allowed to build complex circuits with little cell engineering (Macia et al., 2016).

Additionally, we explored the use of multicellular consortia to build circuits with memory capabilities. To do that, we engineered two populations that produced specific yeast pheromones and inhibited each other (Urrios, Macia, et al., 2016). We showed that when the two populations are mixed together they can stablish memory and remember past inputs.

With these results in mind, we applied multicellular consortia to solve a problem with biomedical relevance. We choose Type I Diabetes as a challenge and built circuits that respond dynamically to glucose homeostasis. As a first step, we built a multicellular

circuit in *S.cerevisiae* that respond to different glucose levels, produced a biological output like insulin or glucagon and respond with a pulse behavior (Urrios, Gonzalez-Flo et al., submitted). While this approach is feasible at non-physiological levels, the results will serve to design the best architecture to build these circuits in mammalian cells.

In summary, these results provide novel insights on the use of multicellularity to build biological circuits.

Additionally, when building complex multicellular circuits with spatial segregation we realized that by having custom microfluidic devices our work could be further exploited. Having this in mind I performed a stay abroad at the Folch lab (University of Washington, Seattle) which specializes in building automated custom microfluidic devices. When I arrived there they were setting up 3D-printing techniques to build microfluidic devices, in this context, I explored the use of 3D-printing to build transparent biocompatible fluidic devices (Urrios, Parra-Cabrera, et al., 2016) which can be further extended to build our custom microfluidic devices.

Los sistemas biológicos recogen información y la computan a diversos niveles. Avances recientes en biología molecular y en tecnologías como la manipulación celular, la ingeniería genética y la síntesis de ADN entre otras, permiten que se puedan diseñar y construir sistemas biológicos, dando lugar a una nueva disciplina biológica, la biología sintética (Manzoni, Urrios et al., 2016).

Actualmente las líneas de investigación están orientadas principalmente a construir circuitos genéticos en células. Sin embargo, construir circuitos complejos dentro de una célula tiene ciertos problemas de escalabilidad como por ejemplo tratar de conectar específicamente múltiples elementos entre sí. Para superar estas limitaciones, se propone el uso de redes multicelulares para construir circuitos (Regot et al., 2011)

En esta tesis doctoral, se ha estudiado el uso de circuitos multicelulares para desarrollar computaciones biológicas en diversos escenarios.

En primer lugar, se ataja el problema de la escalabilidad desarrollando circuitos multicelulares distribuidos en *S.cerevisiae*. El objetivo del estudio es desarrollar un sistema que permita generar circuitos complejos con una mínima manipulación celular. Los resultados obtenidos muestran que minimizando la complejidad de los circuitos intracelulares a puertas lógicas de un input, conectando diferentes células mediante una molécula secretable y segregando las células en distintos ambientes es posible construir circuitos complejos (Macia et al., 2016).

Además, se ha explorado el uso de los circuitos multicelulares para construir circuitos con memoria. Para ello se han modificado dos poblaciones celulares que producen feromonas de levadura específicas y se inhiben mutuamente

(Urrios, Macia, et al., 2016). En este estudio se observa como al mezclar ambas poblaciones se genera un circuito con memoria.

Con estos resultados en mente, se plantea desarrollar circuitos multicelulares que den respuesta a problemas biomédicos, en este caso la Diabetes tipo I. Se han desarrollado circuitos multicelulares en levadura que responden a distintos niveles de glucosa, producen insulina o glucagón y son capaces de responder generando pulsos (Urrios, Gonzalez-Flo et al., submitted). A pesar de que estos circuitos trabajan a niveles no fisiológicos, los resultados son útiles para futuros circuitos en células de mamífero.

En conclusión, los resultados de la investigación generan nuevo conocimiento sobre el uso de la multicelularidad para construir circuitos biológicos.

Adicionalmente, mientras se desarrollaban circuitos multicelulares con segregación espacial se valoró la posibilidad de disponer de dispositivos personalizados de microfluídica para aislar las poblaciones celulares. Por ello realicé una estancia en el Folch lab (University of Washington, Seattle) que se especializan en el desarrollo de dispositivos microfluídicos automatizados, Cuando llegué estaban poniendo a punto técnicas de impresión 3D para producir dispositivos microfluídicos. En este marco, desarrollé técnicas para construir dispositivos fluídicos transparentes y biocompatibles (Urrios, Parra-Cabrera, et al., 2016) que serán útiles para desarrollar futuros dispositivos microfluídicos personalizados.

Els sistemes biològics recopilen informació i la processen a diferents nivells. Els avanços en biologia molecular i d'altres tecnologies com la manipulació cel·lular, l'enginyeria genètica i la síntesis d'ADN, fan que sigui possible dissenyar i construir sistemes biològics. Això ha donat lloc a una nova disciplina anomenada biologia sintètica (Manzoni, Urrios et al., 2016).

Actualment la recerca en aquesta àrea està orientada principalment a construir circuits genètics dintre de cèl·lules. Construir circuits complexes dintre d'una cèl·lula té diversos problemes d'escalabilitat. Per a superar aquestes limitacions, es proposa fer servir l'ús de xarxes multicel·lulars a l'hora de construir els circuits biològics (Regot et al., 2011)

En aquesta tesis doctoral s'estudia l'ús de circuits multicel·lulars per a produir computacions biològiques en diferents escenaris.

En primer lloc, hem adreçat el problema de l'escalabilitat desenvolupant circuits multicel·lulars distribuïts en llevat. Els resultats de l'estudi mostren que minimitzant la complexitat de l'arquitectura intracel·lular dels circuits a una porta lògica d'un únic input, connectant diferents cèl·lules mitjançant una molècula secretable i segregant les cèl·lules en diferents ambients és possible construir circuits complexos (Macia et al., 2016). A més a més, hem explorat l'ús dels circuits multicel·lulars per a construir circuits amb memòria. Per aquest motiu s'han modificat dues poblacions cel·lulars que produeixen feromones de llevat especifiques i s'inhibeixen l'una a l'altre (Urrios, Macia, et al., 2016). En aquest estudi s'observa que la memòria es pot mantenir en un circuit multicel·lular al barrejar les dues poblacions.

Amb aquests resultats, ens hem plantejat dissenyar circuits multicel·lulars que donin resposta a situacions biomèdiques. Ens

hem centrat a la Diabetis tipus I. Els circuits multicel·lulars s'han construït en llevat i responen a diferents nivells de glucosa, produeixen insulina o glucagó i tenen comportaments dinàmics complexes com la generació de polsos a la secreció (Urrios, Gonzalez-Flo et al., submitted). A pesar de que aquests circuits no treballen a nivell fisiològics, els resultats son útils per a dissenyar futurs circuits a cèl·lules de mamífer.

En resum, els resultats de la investigació generen nou coneixement sobre l'ús de la multicel·lularitat per a construir circuits biològics.

Addicionalment, al construir circuits multicel·lulars complexes i distribuir-los espacialment vam valorar la possibilitat de disposar de dispositius de microfluídica personalitzats per a construir els nostres circuits. Poder controlar el entorn extracel·lular amb aquests dispositius permetria estendre el treball previ. Per aquest motiu vaig realitzar una estada al Folch lab (University of Washington, Seattle) on són experts en el desenvolupament de dispositius microfluídics automatitzats. Al arribar estaven posant a punt tècniques d'impressió 3D. En aquest context vaig desenvolupar tècniques per a construir dispositius fluídics transparents i biocompatibles (Urrios, Parra-Cabrera, et al., 2016) que poden ser aplicats per a la creació dels nostres dispositius microfluídics.

PREFACE

Biology works. How? In truth, we do not really know, and that, is amazing. Natural biological systems perform complex computations that include several well-studied responses such as cell division, cell death, or pattern formation in development (Nurse, 2008). Every day that goes by we get a deeper knowledge about how biological systems behave. If we couple this knowledge to advancements in biology and engineering, rational engineering of biological systems becomes a reality. In the past 20 years, synthetic biologists have struggled to build biological systems and there are several stories of success of biological computational devices that perform a variety of functions such as creating edge detectors in *E.coli* (Tabor et al., 2009) or regulating blood glucose homeostasis in diabetic mice (Xie et al., 2016). However, despite these enormous efforts, the results obtained are far from achieving a scalable, robust and predictable life technology.

Current approaches focus mainly on implementing gene expression devices inside a single cell. Inside of a cell there is a lack of spatial distribution between the different elements that perform the computation and this can be a problem in terms of scalability. One of the major issues is the wiring. In electronics (a major source of inspiration for synthetic biology) wires have identical nature and connect different isolated modules or logic gates, however inside of a cell, logic gates and wires are implemented by chemical entities that are not isolated and thus cannot be reused. In this scenario, the wiring is implemented by molecules that have different chemical nature to prevent crosstalk, and there's a limited set of these molecules that despite having different chemical and dynamic properties can be used together. This condition makes circuits too complex and difficult to reuse. To

overcome this we proposed the use of multicellular consortia to build circuits (Regot et al., 2011).

In this PhD thesis, we have developed a LEGO-like strategy for multicellular circuits that can partially avoid the wiring limitation. First, we have combined multicellular circuits with spatial distribution and distributed output and have generated circuits that have low wire requirements but can perform complex computations (Macia et al., 2016). Soon we realized that while our cells were easy to engineer than using other approaches, to achieve the maximum potential we needed a platform with multiple chambers that kept cells isolated while sharing the same environment. To further exploit the potential of multicellular circuits we needed to build custom microfluidic devices, the best alternative for rapid prototyping to produce personalized microfluidic devices is 3D printing technologies, however right now it is a technology still in its infancy (Bhattacharjee et al., 2016). We have started exploring the use of stereolithography to build transparent and biocompatible fluidic devices (Urrios, Parra-Cabrera et al., 2016) and further work need to be done to increase resolution prior assembling a fully functional device.

While we showed that we can build multicellular circuits that integrate different inputs. We wanted to explore if our approach can be used to build circuits that respond based on past and present inputs. We modified our library of cells to build a minimal multicellular memory unit (Urrios, Macia et al., 2016). Memory can confer some advantages to logic circuits and by combining memory units we can create sequential circuits.

To further develop biological computation, we were interested on applying the multicellular approach to a problem with biomedical relevance such as Type I Diabetes. As a first step, we

built a multicellular circuit in *S.cerevisiae* that respond to different glucose levels and produce a biological output with a pulse-like dynamic. (Urrios, Gonzalez-Flo et al., submitted). While this approach works at non-physiological levels, the results will serve to design the best architecture to build circuits in mammalian cells.

In general, this work provides insights of how multicellular circuits work and how the implementation of different architectures give raise to different responses. It also provides guide to new avenues in the areas of sequential circuits, 3D printed microfluidic devices and mammalian biological circuits with biomedical applications.

TABLE OF CONTENTS

INTRODUCTION

1. YEAST AS A MODEL ORGANISM

1.1 *Saccharomyces cerevisiae* as a model organism

Yeast designation includes a subgroup of 1500 different species which belong to the Fungi kingdom. Amongst all yeast species, *Saccharomyces cerevisiae* is the most extensively used as a eukaryotic model organism (Feldmann, 2012a). For the sake of simplicity, yeast and *S.cerevisiae* will be used as synonyms from here on.

Yeast have some convenient properties that make it extremely useful as a model organism (Feldmann, 2012a):

- It is a eukaryotic unicellular organism that can be grown on defined media (liquid culture or agar plates).
- Its life cycle alternates between haploid and diploid phases, and both ploidies can be grown as stable cultures.
- It duplicates by budding, with a duplication time of around 90 minutes in optimal conditions (complete medium, glucose, 30ºC).
- It rapidly adapts and tolerates environmental changes in nutrient, temperature, pH, oxygen concentration and radiation (Hohmann, 2002).
- It is suitable to perform genetic manipulation due to its high-efficiency transformation. Moreover, there is a broad range of selective markers available, both auxotrophic and drug resistance.
- Many cellular functions are conserved from yeast to mammalian cells.

S.cerevisiae cells have an oval shape of 3-6 µm surrounded by a thick cell wall (Figure 1). Cells grow and divide by budding, processes that consume high amounts of energy. The major source of energy production is the conversion of glucose to pyruvate through a process called glycolisis. While glucose is the preferred carbon source, yeast can grow on alternative sugars such as galactose, raffinose, maltose or other carbon sources like ethanol or glycerol. After glycolisis, pyruvate can be further metabolized generating ethanol, CO_2 and 2 molecules of ATP (fermentation) or can be used by the mithocondria in the presence of oxygen to generate a burst of additional molecules of ATP (respiration) (Feldmann, 2012a).

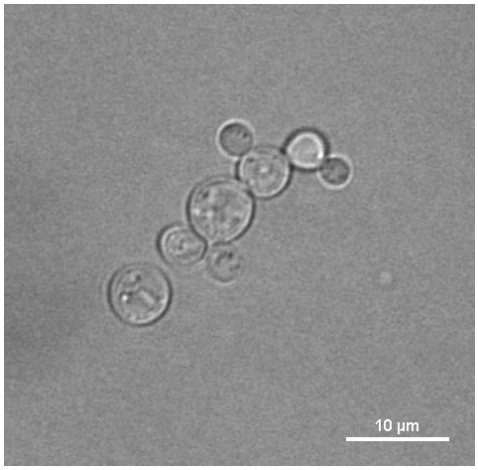

Figure 1. Light microscopy image of *S. cerevisiae* haploid cells.

These properties make yeast a fantastic microorganism for biotechnological processes. From ancient times, yeast have been used for beer or wine production and dough leavening, processes that occur thanks to the fermentation of sugars present in rye or grapes. From 1980s, further understanding of yeast biology has allowed to genetically manipulate yeast cells and turn them into

biofactories for the mass production of peptides, such as insulin (Ferrer-Miralles et al., 2009; Porro et al., 2005) or complex biomolecules such as biofuels (Zhou et al., 2016).

1.2 Yeast adaptation to the environment.

Yeast live in a dynamic environment and have developed several signaling mechanisms to ensure a fast detection and response to different changes such as, pH (Ariño et al., 2010), temperature (Jenkins, 2003), nutrients (Dann et al., 2006), osmotic pressure (de Nadal et al., 2002), or presence of potential mating partners (Dohlman et al., 2006). Among all signal transduction mechanisms, MAPK (mitogen-activated protein kinase) signaling pathways stand out as one of the most studied and robust signaling mechanisms in eukaryotes. Yeast have five MAPK signaling pathways that serve to respond to different stimuli.

1.2.1 MAPK signaling

MAPK pathways are one of the most relevant signal transduction mechanisms in eukaryotes. Eukaryotic cells contain several MAPK pathways that are able to respond to different environmental signals (hormones, growth factors, osmotic pressure,…) in a coordinated manner by modulating gene expression, cell growth, protein homeostasis and other cell functions (Chang et al., 2001; Kyriakis et al., 2001).

The relevance of these signal transduction mechanisms become visible when comparing several eukaryotic cells and finding that these mechanisms are highly conserved from yeast to humans. Moreover, even switching some modules between different species they still preserve some of their functions in the new host (Chen et al., 2007).

MAPK pathways are formed by several proteins or modules that act in a cooperative manner to transduce a signal inside the cell by sequential phosphorylations.

1.2.1.1 Organization and signaling of MAPK pathways

MAPK pathways share a modular organization that consists of a kinase cascade formed by three protein kinases that are activated and phosphorylated sequentially by the previous kinase (Figure 2). Namely, from upstream to downstream, MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK).

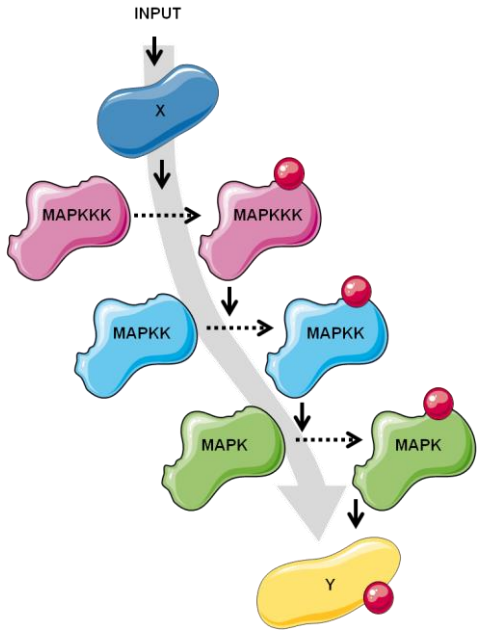

Figure 2. Representation of a canonical MAP kinase pathway. MAPK pathways are composed by three kinases that get sequentially phosphorylated transducing the signal to a final substrate that is phosphorylated.

MAPKKKs act as a Ser/Thr kinase. Activation of MAPKKKs by upstream kinases or other interactors lead to

autophosphorylation through several mechanisms. Activated MAPKKKs phosphorylate MAPKKs on serine and threonine residues leading to their activation.

1.2.1.2 Yeast MAPK pathways

Presently, five different MAPK pathways have been well characterized in *S.cerevisiae* (Hohmann, 2002; Qi et al., 2005) (Figure 3): Pheromone pathway (Fus3), Filamentous/invasive growth pathway (Kss1), High osmolarity growth pathway (Hog1), Cell wall integrity pathway (Slt2) and Spore wall assembly pathway (Smk1).

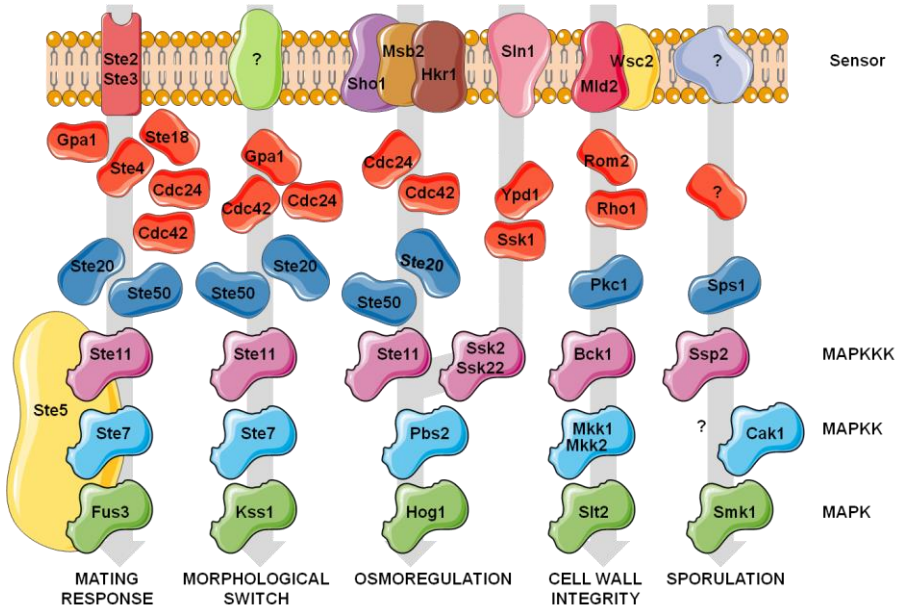

Figure 3. *S.cerevisiae* MAPK pathways. *S.cerevisiae* has five MAPK pathways: mating response, morphological switch, osmoregulation, cell wall integrity and sporulation. MAPKKK are depicted in pink, MAPKK in blue and MAPK in green for each pathway.

The signal starts by activation of receptors at the membrane that trigger a cascade of events (phosphorylations, inhibitions,

complex formation…) leading to the activation and phosphorylation of a specific MAPKKK that will phosphorylate a MAPKK which will phosphorylate the final MAPK. Yeast have five MAPKs (Fus3, Kss1, Hog1, Slt2, Smk1) that are controlled by four well described MAPKKs (Ste7, Pbs2, Mkk1/Mkk2) which in turn are controlled by four MAPKKKs (Ste11, Ssk2/Ssk22, Bck1) (Chen et al., 2007).

MAPKKKs and MAPKKs can control more than one MAPK. To guarantee a specific respond to different stimulus and the activation of a specific MAPK it is necessary to have accessory proteins that coordinate these elements. All these elements and their interactions form a high complex signaling network that prevent crosstalk and ensure specificity (Qi et al., 2005). Additionally, eukaryotic organisms have developed other strategies to ensure the correct signal transduction, such as, docking interactions, pathway inhibitions or requirements for scaffold proteins (Saito, 2010).

1.3 Yeast life cycle

S.cerevisiae have two different haploid cell types and one diploid cell type. MATa (a-cell) and MATα (α-cell) are the haploid types, these two haploid types can mate and fuse giving rise to the MATa/MATα diploid (a/α-cell). Yeast has two modes of reproduction: (i) vegetative growth by asexual budding in both haploid and diploid types, or (ii) mating of haploid cells of opposing types generating a diploid cell. For mating, haploid cells need to find a close mating partner. Each haplotype produces and secretes a specific diffusible pheromone. If a cell of an opposite type detects high concentrations of the opposite pheromone it activates the pheromone MAPK pathway. The synchronized activation by two close opposite cells of the pathway, ends up in the mating and

fusion of the cells giving rise to a diploid cell (a/α cell). Upon starvation, a/α cell can sporulate and undergo meiosis generating four haploid cells (Feldmann, 2012a) (Figure 4).

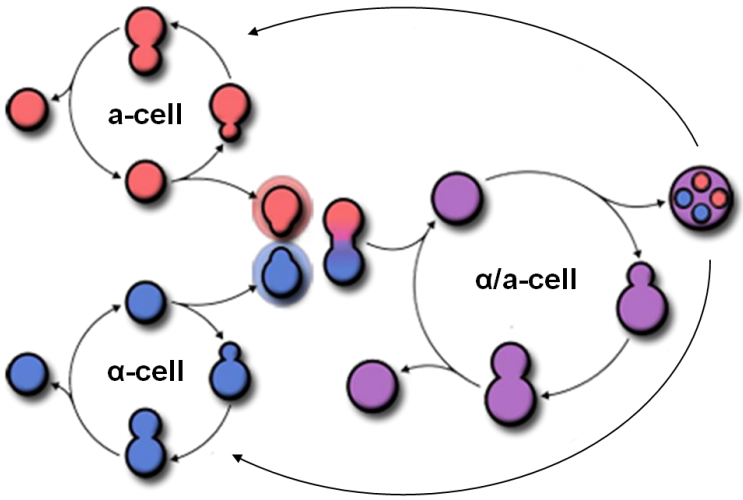

Figure 4. Life cycle of *S.cerevisiae*. *S.cerevisiae* can grow and divide in both haploid and diploid types. There are two haploid types: MATa (a-cell) and MATα (α-cell) that can mate and generate a diploid cell (a/α-cell). Diploid cells can sporulate and generate four haploid cells.

1.3.1 Yeast mating types and pheromones

Mating between a-cells and α-cells is initiated by secretion of diffusible pheromones that are recognized by specific receptors on the opposite cell type. α-cells produce and secrete α-factor a tridecapeptide (WHWLQLKPGQPMY) pheromone that is recognized by Ste3, a GPCR located on the surface of a-cells (Hagen et al., 1986). a-cells release a-factor to the media, a farnesylated dodecapeptide (YIIKGVFWDPAC (farnesyl)OCH_3) pheromone that is recognized by Ste2, a GCPR expressed in α-

cells (Konopka et al., 1988). The binding of the pheromone to the receptors triggers a signaling response through a MAPK pathway, the pheromone pathway, that induces several cellular changes, such as cell cycle arrest in G1 (McKinney et al., 1995), cell membrane polarization towards the pheromone gradient (shmoo formation) (Segall, 1993) or changes in gene expression (about 200 genes - 3% of the genome (Zheng et al., 2010). Several components involved in this signaling process are called STE, this name comes from the word *sterile* and it is due to the earlier methods used for the identification of these components. Most of the components were identified in genetic screenings where some mutations render cells with a sterile phenotype that impairs them to mate.

1.3.2 The pheromone pathway

1.3.2.1 Pheromone production

In *S.cerevisiae*, α-factor is encoded by *MFα1* and *MFα2 in* α-cells while a-factor is encoded by *MFA1* and *MFA2* genes in a-cells. Despite their functional equivalence, their structure, secretion and biogenesis are different (Figure 5).

Mature a-factor is synthesized from a precursor of 36 amino acids that contains a long N-terminal extension and a C-terminal CAAX motif. Initially, a-factor precursor undergoes several CAAX modifications (farnesylation and carboxymethylation), and then the N-terminal extension gets cleaved and a-factor shuttles to the membrane and exits the cell via Ste6. After export, a-factor diffuses and interacts with Ste3 receptor on the surface of α-cells triggering the pheromone pathway. The mature form of a-factor is highly hydrophobic (Michaelis et al., 2012).

Mature α-factor is synthesized from a precursor of 165 amino acids that contains a long N-terminal extension (pre-pro leader sequence) and four tandem copies of 13 amino acids separated by spacer sequences that can be cleaved. α-factor precursor translocates to the endoplasmic reticulum where the N-terminal sequence gets cleaved and is transported to the Golgi. In the Golgi, three proteolytic cleavage steps are required, mediated by Kex1, Kex2 and Ste13, yielding four copies of mature α-factor. Secretory transport vesicles containing α-factor are formed in the Golgi. These vesicles fuse with the plasma membrane releasing α-factor to the media. After export, α-factor diffuses and interacts with Ste2 receptor on the surface of a-cells triggering the pheromone pathway. The mature form of α-factor is hydrophilic (Naider et al., 2004).

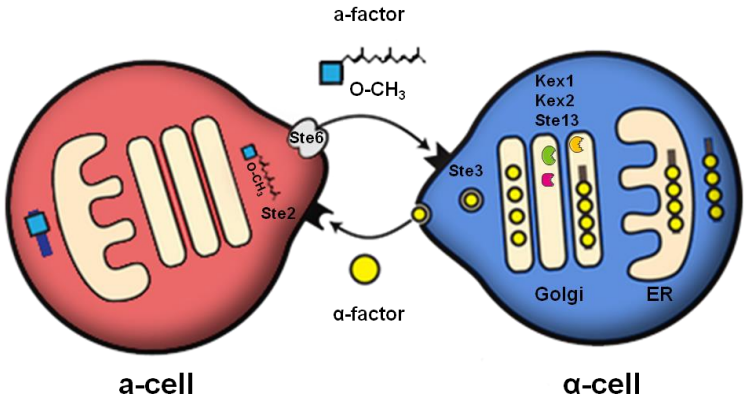

Figure 5. Pheromone production and secretion in *S.cerevisiae*.
α-cells secrete α-factor though the ER and Golgi pathway. a-cells produce a-factor which is secreted through Ste6. Both pheromones are detected by either Ste2 or Ste3 receptors on the opposite cell type (Michaelis et al., 2012)

1.3.2.2 Pheromone sensing

Ste2 and Ste3 are seven-transmembrane receptors of the GPCR family, associated with a heterotrimeric G-protein complex that acts as the starter of the pheromone pathway (Bardwell, 2005). The G-protein complex is formed by Gpa1 (α-subunit), Ste4 (β-subunit) and Ste18 (γ-subunit). Pheromone binding by the receptor induces GDP to GTP exchange in Gpa1, causing its dissociation from the hererotrimer. Ste4-Ste18 dimer binds to the membrane and orchestrates the assembly of an activating complex formed by Cdc42, Ste20, Ste11, Ste50 and Ste5. Ste5 acts as a scaffold recruiting the pheromone MAPKKK (Ste11), MAPKK (Ste7) and MAPK (Fus3) (Ramezani-Rad, 2003) (Figure 6).

Cdc42 activates Ste20 which in turn phosphorylates Ste11 triggering the MAPK cascade. Ste11 acts as a MAPKKK and phosphorylates Ste7 MAPKK which activates Fus3 MAPK (Dowell et al., 1998). Activated Fus3 orchestrates several cellular responses, for example, activates Ste12 that acts as a transcription factor for pheromone-specific genes (*FUS1, FIG1, FIG2, PRY3…*) (Zheng et al., 2010); imposes G1 cell cycle arrest through Far1/Cdc28 (McKinney et al., 1995); promotes polarized growth, shmoo formation (Segall, 1993); and changes in cell's plasma membrane, cell wall and nucleus, preparing the cell for cell fusion (Merlini et al., 2013).

The activity of the yeast pheromone pathway is controlled by several negative feedback loops that facilitate cell recovery after exposure to the pheromone (Ma et al., 1995). Some of these negative feedback loops are mediated by the following mechanisms: a-cells have developed an extracellular method for desensitization by releasing Bar1, a pepsin-like protease that degrades α-factor. Moreover, *BAR1* expression is induced after

pheromone stimulation (Manney, 1983). At the receptor level, Fus3 phosphorylates the receptor, inducing its endocytation (Chen et al., 1996). At the G-protein level, Sst2 mediates the hydrolysis of the Gpa1p-bound GTP. Hydrolysis of GTP, deactivates Gpa1 which binds to Ste4-Ste18 complex and form the inactive heterotrimer, weakening the signal (Dohlman et al., 1996). Expression of *SST2* is induced after pheromone treatment and Sst2 stability is enhanced after phosphorylation by Fus3 MAPK (Garrison et al., 1999). Other mechanisms, involve phosphatases that counteract the signal, for example, Msg5 phosphatase regulates Fus3 activity by desphosphorylation (Doi et al., 1994).

Pheromone stimulation can activate the filamentous growth MAPK Kss1 even in the absence of Ste5 (Gagiano et al., 2002; Ma et al., 1995). Absence of both Fus3 and Kss1 make cells sterile, however having either one or the other allows mating, pointing out that these two MAPKs may be redundant for some functions. In order to ensure pheromone pathway specificity, cells have developed several mechanisms: like a nuclear increase of Fus3 levels after pheromone treatment (Blackwell et al., 2007), higher affinity of Fus3 to substrates like Far1 (Bardwell et al., 1996), or specific Fus3 degradation of Tec1, a transcription factor of the filamentous growth pathway (Brückner et al., 2004), among others (Ma et al., 1995).

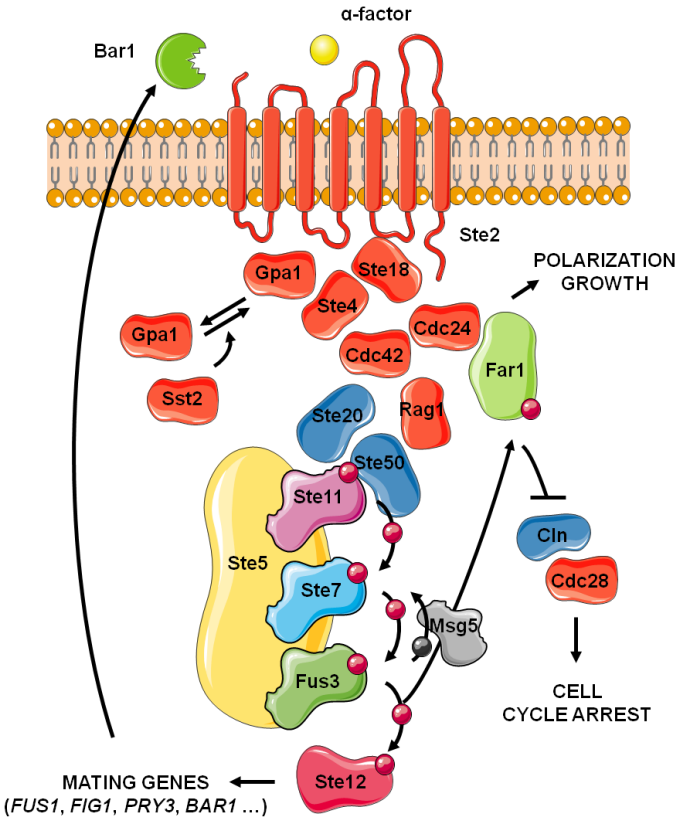

Figure 6. Pheromone sensing in *S.cerevisiae*. α-factor binds Ste2 receptor and triggers the disassembly of the downstream G-protein (Gpa1, Ste4 and Ste18). Ste4-Ste18 dimer binds to the membrane and recruits intermediary proteins: Cdc24, Cdc42, Rag1, Ste20, Ste50, Ste5. Ste5 acts as a scaffold protein recruiting Ste11 (MAPKKK), Ste7 (MAPKK) and Fus3 (MAPK). Ste4-Ste18 causes Cdc42 phosphorylation that phosphorylates Ste20 and causes the sequential phosphorylation of Ste11, Ste7 and Fus3. Fus3 phosphorylates different substrates causing the pheromone pathway response. It phosphorylates Ste12 that will induce mating genes, and phosphorylates Far1 that will produce cell cycle arrest in G1 phase. There are some negative feedback loops like Bar1 protease that degrades α-factor, Msg5 phosphatase that dephosphorylates Fus3 or Sst2 that recovers Gpa1.

1.4 Glucose metabolism

Glucose is the primary carbon source for *S.cerevisiae*. To maximize its use, yeast have developed mechanisms to sense glucose availability and optimize its uptake over a broad range of concentrations. There are two main glucose-sensing pathways, the Snf1/Snf4 pathway and the Snf3/Rgt2 pathway (Johnston et al., 2005) (Figure 7).

Other than sensing, yeast has specialized on glucose uptake and possess 18 different hexose transporters with different affinities and capacities to maximize glucose uptake at different ranges of extracellular glucose levels (Wieczorke et al., 1999).

Six of them (Hxt1,2,3,4,6,7) are characterized as glucose transporters (Özcan et al., 1999), one (Gal2) is a galactose transporter (Tschopp et al., 1986), and the others (Hxt5 and Hxt8-17) are related to transport of fructose and mannose (Wieczorke et al., 1999).

Once glucose gets inside the cell it is metabolized to pyruvate through glycolisis. At this point pyruvate can face two fates, fermentation or respiration (Johnston et al., 2005). Pyruvate can be transformed to CO_2 and ethanol producing 2 ATP molecules (fermentation), or in the presence of oxygen, pyruvate can be converted to CO_2 and water by the mitochondria generating a burst of ATP (36 ATPs) (respiration). *S.cerevisiae* prefers to do fermentation even if oxygen is abundant. This effect is called Crabtree effect (Crabtree, 1929). A similar effect is seen in tumor cells known as Warburg effect (Warburg et al., 1927).

1.4.1 Major pathways of glucose regulation

Glucose addition triggers massive transcription changes; about 20% of *S.cerevisiae* genes experience a 3- fold change in expression, and 40% at least a 2-fold change (Feldmann, 2012b; Hedbacker et al., 2008; Yin et al., 2003).

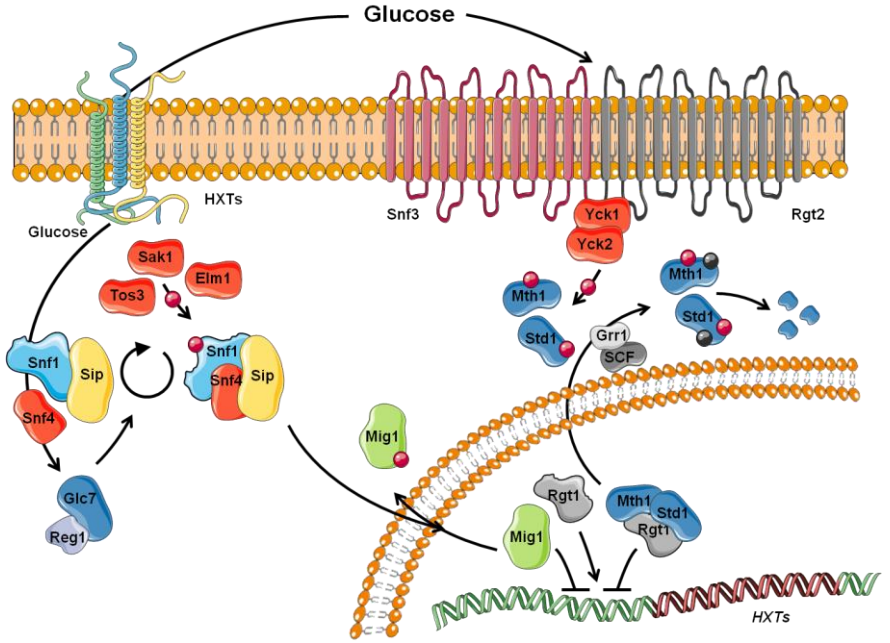

Figure 7. Major pathways of glucose regulation in *S.cerevisiae*. There are two main glucose-sensing pathways: the Snf1/Snf4 pathway and the Snf3/Rgt2 pathway. Snf1/Snf4 controls gene glucose-repression through Mig1 action. In the absence of glucose, Snf1 is activated and phosphorylates Mig1 making it to leave the nucleus and derepressing several genes. In the presence of glucose, Glc7/Reg1 complex deactivates Snf1. Snf3/Rgt2 senses extracellular glucose through Snf3/Rgt2 receptors. This causes the release of Rgt1 from the inhibitory complex Mth1-Std1, allowing the induction of some *HXT* genes.

1.4.1.1 The Snf1/Snf4 pathway

The Snf1/Snf4 system is required for cell adaptation and growth in the presence of extracellular of other on non-glucose carbon sources. The Snf1 kinase (AMPK in mammalian cells) complex is a heterotrimeric complex formed by Snf1, the catalytic α-subunit, Snf4p, a regulatory subunit, and three interchangeable β-subunits that seems to determine substrate specificity. Snf1 is activated by phosphorylation by upstream kinases Sak1, Tos3 and Elm1 in response to glucose limitation (Hong et al., 2003; Nath et al., 2003), and inactivated by Reg1/Glc7 phosphatase (Sanz et al., 2000; Tu et al., 1995).

In high-glucose conditions, the catalytic domain of Snf1 is autoinhibited by forming a loop between the N-terminal catalytic domain and the C-terminal regulatory domain (Jiang et al., 1996). In low-glucose conditions, Snf1 catalytic domains bind to Snf4, reducing its autoinhibition and allowing Snf1 to phosporylate different substrates.

Mig1 is a transcriptional repressor that binds to many glucose-repressed genes (Hardie et al., 1998). In the presence of glucose Mig1 is localized in the nucleus and quickly moves to the cytosol when glucose is depleted. This action is mediated by Snf1 complex. Glucose depletion activates Snf1 which in turn phosphorylates Mig1 (Östling et al., 1998; Treitel et al., 1998) forcing it to leave the nucleus (DeVit et al., 1999).

In addition to deactivation of the Mig1 repressor, the Snf1 complex activates the transcriptional factors Cat8 and Sip4 (Hedges et al., 1995; Vincent et al., 1998), and participates in multiple processes such as regulation transcription and translation, glycogen and lipid synthesis and general stress responses (Kayikci et al, 2015).

1.4.1.2 The Snf3/Rgt2 pathway

The Snf3/Rgt2 pathway optimizes yeast growth at different extracellular glucose levels. The pathway starts with two transmembrane receptors that act as glucose sensors by binding glucose and triggering a signaling cascade (Marshall-Carlson, Celenza, Laurent, & Carlson, 1990; Moriya & Johnston, 2004).

Snf3 has high affinity for extracellular glucose, while Rgt2 has low affinity (Ozcan et al., 1996). Snf3 is required for expression of the glucose transporters *HXT2* and *HXT4* in low glucose. By contrast Rgt2 plays a critical role in the glucose transporter *HXT1* induction at high levels glucose (Ozcan et al., 1998). Yck1p/Yck2 interacts with the C-terminal intracellular domains of Snf3/Rgt2. Activation of the receptors by glucose leads to the activation of Yck1/Yck2 (Johnston et al., 2005; Moriya et al., 2004). The targets of activated Yck kinases are Std1 and Mth1 (Lafuente et al., 2000). The phosphorylation of Std1 and Mth1 cause their ubiquitination and degradation by the proteasome (Li et al., 1997).

In the absence of glucose, Rgt1 interacts with the co-repressors Std1 and Mth1, and form a complex that represses the expression of several glucose transporter genes. Upon glucose stimulation, degradation of Std1 and Mth1 turns Rgt1 into an activator (Mosley et al., 2003).

Repression by both Rgt1 and Mig1 also require association with Ssn6 and Tup1 (Ozcan et al., 1995), which work as a general repressor of gene expression (Keleher et al., 1992; Trumbly, 1992).

1.4.2 Hexose transporters

Glucose is transported inside the cell using different glucose transporters encoded by the *HXT* genes. These transporters have different affinities and transport capacities, and their expression is regulated by the extracellular glucose levels (Ozcan et al., 1995; Özcan et al., 1999)

None of these transporters alone is essential for growth on glucose. However a strain lacking *HXT1-7* genes cannot grow on glucose, meaning that these genes encode for functional glucose transporters that share functional redundancy (Boles et al., 1997; Liang et al., 1996; Reifenberger et al., 1997). On one hand, *HXT2*, *HXT6*, or *HXT7* allow cells to grow on 0.1% glucose (low) suggesting that they produce high-affinity transporters. On the other hand, *HXT1*, *HXT3*, or H*XT4* allow cells to grow on glucose concentrations higher than 1% (high), suggesting that they produce low-affinity glucose transporters (Reifenberger et al., 1997). Except for *HXT4*, the other *HXT* genes seems to be regulated in response to glucose according to their glucose transport affinity, the lower the glucose the higher expression of high-affinity transporters (Bisson et al., 1993; Boles et al., 1997; Ozcan et al., 1995).

HXT3 expression is not affected by glucose levels, *HXT2-4* and *HXT6-7* are expressed at low glucose levels and *HXT1* is expressed at high glucose levels. In detail: *HXT3* expression is induced about 10-fold in either low or high glucose concentrations (Ozcan et al., 1995). Deletion of *RGT1* causes constitutive expression of *HXT3*.

HXT2 and *HXT4* are induced 10-fold by low glucose levels (0.1%) and by raffinose. If there is no glucose, Rgt1 binds *HXT2* and *HXT4* promoters repressing their expression. At high glucose

levels, Mig1 represses their expression (Nehlin et al., 1991; Ozcan et al.,1996). At low glucose levels (~0.05 to ~0.4%) both repressors are inactive and *HXT2* and *HXT4* are expressed.

HXT6 and *HXT7* expression are repressed by high glucose levels and have a higher expression when grown in the absence of glucose (Boles et al., 1997; Liang et al., 1996). Snf3 is required for glucose repression (Ozcan et al., 1995).

HXT1 is induced about 300-fold at high glucose levels (4%). Rgt1 represses *HXT1* in the absence of glucose while activates its transcription at high glucose (Ozcan et al., 1996). In *rgt1* mutants another regulatory mechanism mediates *HXT1* expression by high levels of glucose. Additionally, *HXT1* expression is induced in high osmolarity conditions (1M NaCl) (Hirayarna et al., 1995). This expression requires Hog1, a MAPK required for hyperosmotic shock adaptation (Brewster et al., 1993).

2. SYNTHETIC BIOLOGY

2.1 What is synthetic biology?

Synthetic biology is a research field which aims to design, develop and implement novel biological functions by combining the knowledge of different engineering and biology-related disciplines.

The field originated in 2000 with the early attempts to build synthetic genetic circuits in bacteria, a toggle switch and an oscillator (Elowitz et al., 2000; Gardner et al., 2000). From that time forward many synthetic biological circuits with different applications have been developed in different organisms from bacteria to mammalian cells. These circuits are able to detect different environmental stimuli, store information and make decisions (Manzoni et al., 2016). The field has experienced a fast growth and now has become an important part of modern biology.

2.2 Biological computation

Cells constantly integrate extracellular and intracellular information (inputs) in order to make decisions, generate a response (output) and keep cellular fitness (Nurse, 2008). This process of controlling information, transforming inputs into outputs, can be understood as cellular computation. Computation can be classified in two big blocks according to the relation between inputs and outputs: analog or digital. Analog circuits sense and produce gradual signals while digital circuits work with binary signals. Cells implement both types of computation in different processes (Macía et al., 2009; Sauro et al., 2013).

On the other hand, electronic circuits work mainly through digital computations, producing binary electric signals; either

voltage is low (“0”) or high (“1”). These two states can be used to represent numbers that can be combined to perform complex computations. While the mechanisms governing cellular computations are still elusive, computations in electronic circuits are well understood.

Getting inspiration from electronics, several synthetic circuits have been built in cells, both implementing digital (Purcell et al., 2014; Roquet et al., 2014) and analog responses (Daniel et al., 2013; Farzadfard et al., 2014). Unlike electronic circuits where the computing modules are physically isolated and connected with wires, in a cellular context this architecture cannot be implemented since all the components share the same environment. To ensure a good communication between the different modules, different specific wiring molecules are needed in order to prevent crosstalk and guarantee a good communication. One of the problems is that these wiring molecules are different and have different properties and since the same molecule cannot be reused, the number of molecules needed increase with the complexity of the circuit. Upon all these, it is essential to keep in mind that the cells are a dynamic environment constantly changing due to the necessity to grow and adapt to the extracellular environment.

2.2.1 Digital biological synthetic circuits

Digital biological synthetic circuits behave with a switch-like behavior. They sense stimuli that can both be high “1”, when the input is over a threshold, or low “0”, below the threshold, and produce a specific response accordingly.

Digital devices are built by assembling simple computing units called logic gates. Logic gates are basic modules that accept binary inputs (0 or 1) and transform them into a binary output

following Boolean rules. Logic gates are the basic building blocks to build more complex digital logic circuits.

2.2.1.1 Basic Boolean logic

Boolean logic is a set of mathematical rules that describes the possible relations between inputs and outputs with binary numbers (0 or 1) (Figure 8).

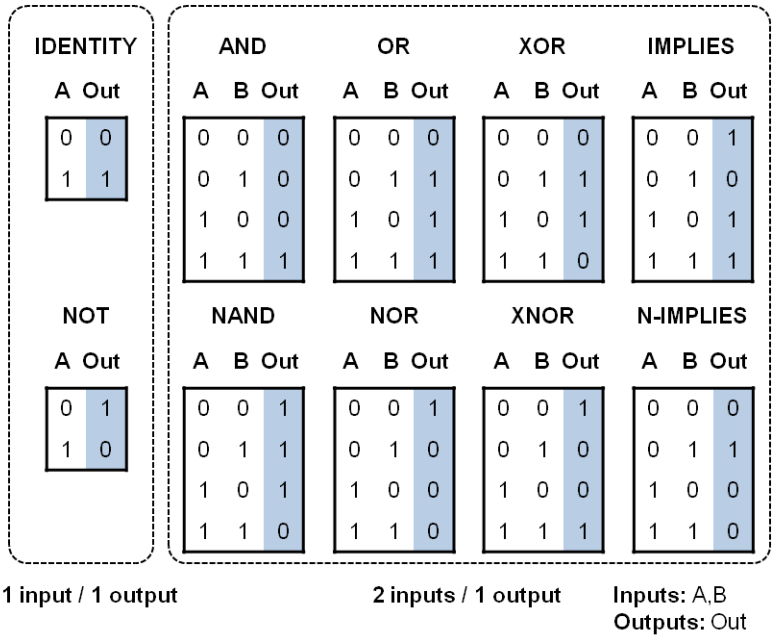

Figure 8. Truth table of main Boolean logic operators. Truth table of the main unary and binary Boolean logic operators.

For one input-one output there are 2 possible combinations or logic gates: IDENTITY and NOT. IDENTITY gate gives an output of 1 only when the input is 1, it only produces an output in the presence of an input. In biology its behavior is like an inducible promoter that induces transcription in the presence of the inducer. NOT gate gives and output of 1 only when the input is 0. In biology the behavior is like a repressible promoter that only is expressed in

the absence of the repressor. For two input-one output functions there are 8 major combinations: AND, OR, XOR, IMPLIES, NAND, NOR, XNOR, N-IMPLIES. One graphical way to represent the relations of the combination of inputs and the output is by using a truth table (Figure 8)

2.2.1.2 Implementing logic gates in biology

Several of these logic gates can be found in nature, for example the bacterial Tet OFF system behave as one input-one output logic gates (Gossen & Bujard, 1992). In the absence of doxycycline (input) the promoter is on (output), but when doxycycline is present the promoter is repressed. An example of a more complex natural logic gate is the yeast *GAL1* promoter. *GAL1* promoter behaves like a N-IMPLIES gate. In the presence of galactose, the *GAL1* gene is induced; however if glucose is present this induction is prevented. Thus, *GAL1* is expressed when galactose is present (1) and glucose is absent (0), a behavior expected for an N-IMPLIES logic gate (Johnston et al., 1999; Lohr et al., 1995).

Besides natural logic gates, several synthetic logic gates have been implemented by different means such as using novel and modified transcription factors (Elowitz et al., 2000; Gardner et al., 2000), engineering RNA regulators (Lucks et al., 2011; Win et al., 2008), rewiring signaling pathways (Furukawa et al., 2013, 2015) or establishing multicellular consortia (Regot et al., 2011; Tamsir et al., 2011a).

2.2.1.3 Multiple-input synthetic circuits

Complex logic circuits that respond to different inputs have been successfully engineered in different organisms using some of the aforementioned logic gates (Manzoni et al., 2016). For example, a circuit known as HELA cancer cell classifier is able to detect the presence of 6 pathological microRNAs molecules and triggers apoptosis (Xie et al., 2011).

There are two main strategies to integrate several inputs in a synthetic circuit, by either connecting simple logic gates or by using more complex multi-input logic gates (Figure 9). Multi-input logic gates are more difficult to engineer and to test since it requires to develop a biological construct, DNA, protein, … that can respond to different specific inputs (Figure 9A), not always with the same behavior, to produce an output. Despite some success in building multi-input logic gates (Bonnet et al., 2013a), the preferred form to build complex circuits is by orthogonally layering different logic gates (Figure 9B). In other words, make a complex genetic circuit by simple genetic circuits where the output of a logic gate serves as the input of the following one (Moon et al., 2012).

Building complex circuits by layering simple logic gates is easier because the architecture of the logic gates is simple and is simple to engineer, to test and to tune. However, while connecting different gates with wires in electronics is easy, doing it inside of a cell is difficult. This is mainly because in electronics logic gates are spatially isolated, however inside of a cell, molecules and logic gates often share the same environment. To prevent crosstalk when connecting different biological gates, different molecules needs to be used. This is a limiting factor to scale up circuits (Furukawa et al., 2013; Macía et al., 2012). One way to address this scalability problem is to isolate the logic gates in different cell

types and use multicellular consortia together with spatial segregation to perform the computations (Macia et al., 2016).

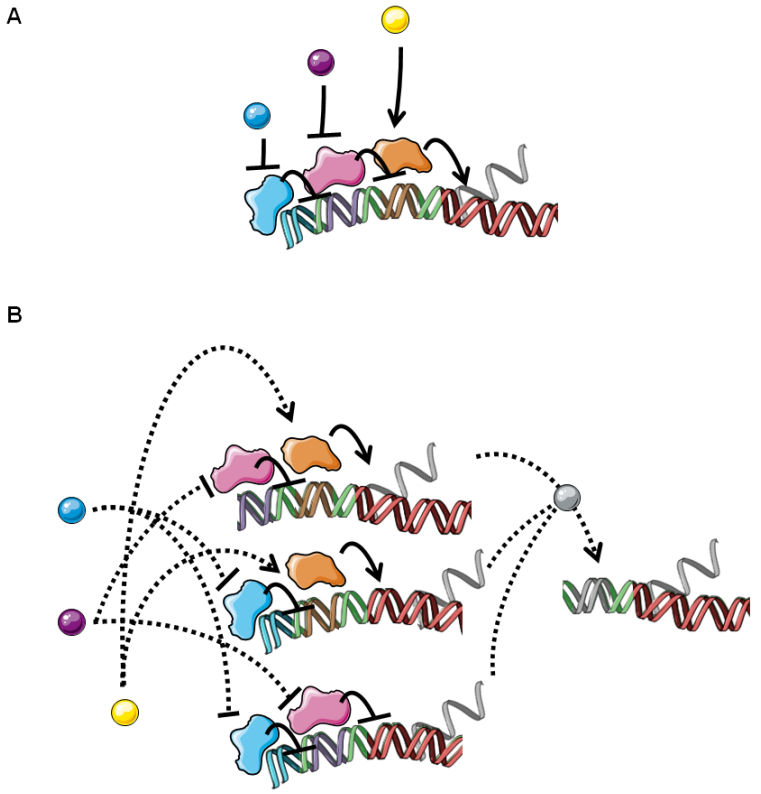

Figure 9. Multi-input synthetic circuits. (A) Example of a multi-input logic gate where the transcription of the promoter will be dependent only on the combination of the inputs and if they are activators or repressors. (B) Schematic representation of a circuit with different logic gates that are orthogonally layered. The transcription of the genetic circuits on the left depend on the combination of inputs and their activity to produce a wiring molecule that will act on a second promoter that will produce the final output.

When building multi-input circuits, the architecture is a critical step. One specific function can be implemented by different combinations of logic gates. Thus, it is crucial to study in advance

which set of logic gates are going to be implemented that minimizes and simplifies the engineering of the biological parts.

2.2.1.4 Digital memory and sequential circuits

There is a huge interest in building combinatorial circuits that can integrate several inputs and respond accordingly. However not everything is about how to integrate the maximum amount of inputs. How to keep the response stable through time is equally important.

Memory circuits can store present inputs and retain the information even if the input is removed. Memory is at the core of many biological systems (Macía et al., 2009) such as cell-fate determination (Chickarmane et al., 2006; Ferrell et al., 1998), cell cycle regulation (Sha et al., 2003) or epigenetic regulation (Li, 2002). So far, synthetic memory circuits have been built following 2 approaches: active transcriptional positive (Figure 10A) or double negative feedback loops (Gardner et al., 2000) or selective expression of recombinases (Isaacs et al., 2003) (Figure 10).

The toggle switch (Gardner et al., 2000) is a synthetic memory circuit built in *E.coli.* Two synthetic promoters produce repressors that inhibit each other establishing a double negative feedback loop (Figure 10B). This circuit can establish memory fast, as soon as one of the repressors surpasses a threshold. However, it requires constant production of the repressors to maintain the memory state which can impact on the bioenergetics of the cell. Additionally, changes in the cell such as cell growth can affect the levels of the repressor and affect the memory state. These limitations make this approach unsuitable to build bigger and complex gene circuits.

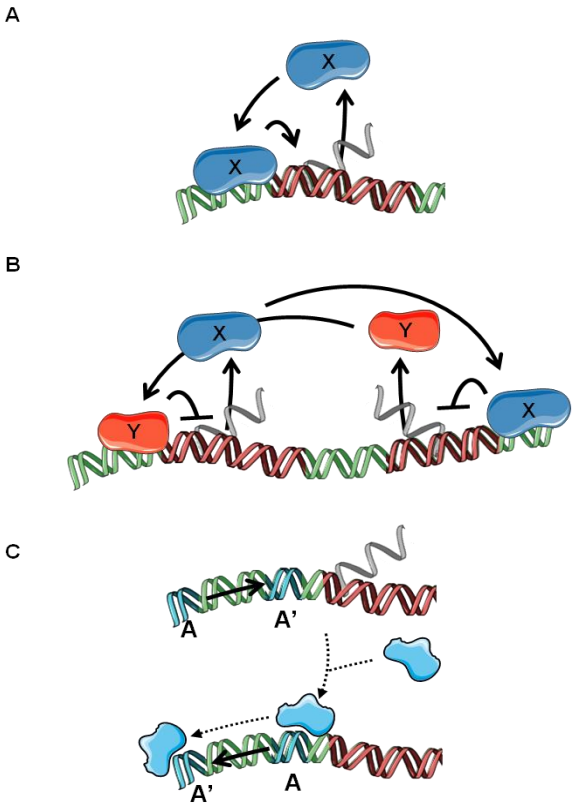

Figure 10. Different synthetic memory circuits. Transcriptional memory circuits can be engineered by implementing:(A) A positive feedback loop where X triggers its own expression. (B) A double negative feedback loop where X inhibits Y expression and Y inhibits X expression. (C) Memory can be encoded in the DNA. A promoter region flanked by recombinase target sequences triggers the expression of a gene. When the recombinase is present the promoter region flips pointing outside the gene body and the gene is no longer expressed.

Recombinases can target DNA regions and excise or invert them altering gene expression. Recombinases have been used to establish stable memory in DNA (Bonnet et al., 2012; Wang et al.,

2011) by using promoter regions flanked by recombinases recognition sites. A unidirectional promoter flanked by recombinase recognition sites can induce a gene. Presence of the recombinase, inverts the promoter, shutting down the expression of the gene (Figure 10C). This method although being slower than transcriptional memory it has a great temporal stability without generating a metabolic burden to the cell.

Memory confers some advantages to logic circuits. For example, adding memory to a circuit allow the circuit to remember the presence of an input that can be no longer present. This can reduce costs in some industrial bioprocesses by reducing the amount of inducer needed to keep the circuit working(Nevoigt, 2008).

2.3 Engineered biological modules for yeast

Among the aforementioned strategies, signaling pathway rewiring and transcriptional regulators are one of the most used strategies to build synthetic circuits in yeast. In this section, the rewiring of the pheromone pathway, the galactose regulon and the use of human hormones and *E.coli* proteins will be explained.

2.3.1 Pheromone pathway rewiring

Yeast cells have been used to build multicellular circuits (Regot et al., 2011). In this context, the use of wiring molecules between cells is critical. The use of pheromones has been implemented by which one cell senses different inputs and produce or not a secretable pheromone (α-factor) which is detected by other cells which in turn can detect or not other inputs. In this setting, α-factor works as a secretable wiring molecule. Several modifications on the pheromone pathway can modify the behavior of the circuits.

For example, substitution of the receptor Ste2 by its cognate receptor in the species *Candida albicans* or *Debaryomyces hansenii* will render cells no sensitive to *S.cerevisiae* α-factor but to the α-factor-like peptides of *C.albicans* (GFRLTNFGYFEPG) or *D. hansenii* (FHWMTYRFFQPNL) respectively. This strategy of using receptors and pheromones from other yeast species that do not generate crosstalk can be used to layer different logic gates.

Other modifications of the circuit can include the secretion of Bar1 protease to degrade the wiring molecule (α-factor) (Urrios, Macia, et al., 2016), or the use of MAPK mutants to allow or abolish the signaling cascade triggered by pheromone detection (Regot et al., 2011).

2.3.2 The galactose regulon

Yeast cells are able to sense the presence of galactose in the media and activate the *GAL* genes to metabolize galactose (Lohr et al., 1995) only if glucose is absent (Mark Johnston, 1999) (Figure 11).

Gal4, binds to the upstream activating sequences (UAS) in the *GAL* promoter region and induces transcription. In the absence of galactose, Gal80 binds Gal4 and blocks the activation. When galactose is present Gal3 inhibits Gal80 facilitating the Gal4 mediated transcription of the *GAL* genes. In the presence of glucose, Mig1 represses Gal4 activity (Nehlin et al., 1991).

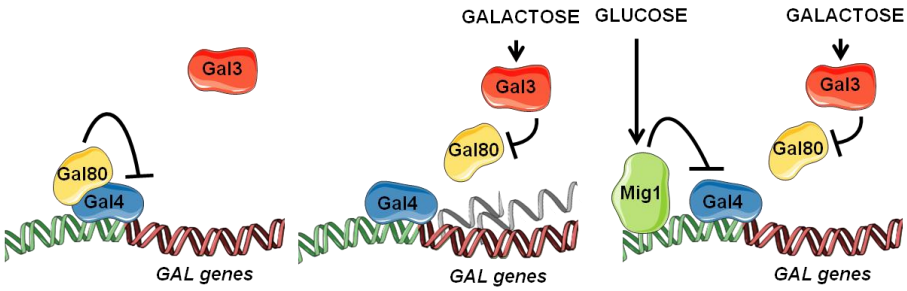

Figure 11. Schematic regulation of *GAL* genes. In the absence of glucose or galactose, Gal80 represses the activity of Gal4, inhibiting the expression of *GAL* genes. When galactose is added, Gal3 inhibits Gal80, and Gal4 induces the expression of *GAL* genes. Addition of glucose, activates Mig1 that inhibits Gal4 activity shutting down the expression of *GAL* genes.

2.3.3 Detection of human hormones in yeast

A synthetic hybrid receptor is able to activate the *GAL* genes in the presence of 17β-Estradiol even if glucose is present (Takahashi et al., 2008). This chimeric receptor contains a Gal4 DNA binding domain, an estradiol receptor domain (ER) and a VP16 transcriptional activator protein. In basal conditions, the protein is sequestered in the cytosol by chaperones that bind the ER domain. When 17β-Estradiol is present in the media, it binds to the ER domain causing the release of the chaperones and producing the nuclear translocation of the Gal4DBD-ER-VP16. The Gal4DBD targets the chimeric protein to the *GAL* UAS, then VP16 domain recruits RNA pol II which trigger transcription (Figure 12).

A similar approach was used to create a chimeric protein able to respond to progesterone and aldosterone. In this construct, the ER domain was substituted by the ligand binding domain of progesterone receptor (hPR from aminoacid 655 to 933) or the

ligand binding domain of aldosterone (hAR, from aminoacid 705 to 984) (Macia et al., 2016).

Similar to estradiol, progesterone or aldosterone, yeast is able to sense dexamethasone if the whole glucocorticoid receptor (hGR) is expressed in yeast. hGR remains in the cytoplasm under basal conditions, addition of dexamethasone cause its translocation to the nucleus where can bind HERE sequences and trigger transcription (Macia et al., 2016; Miller et al., 2010).

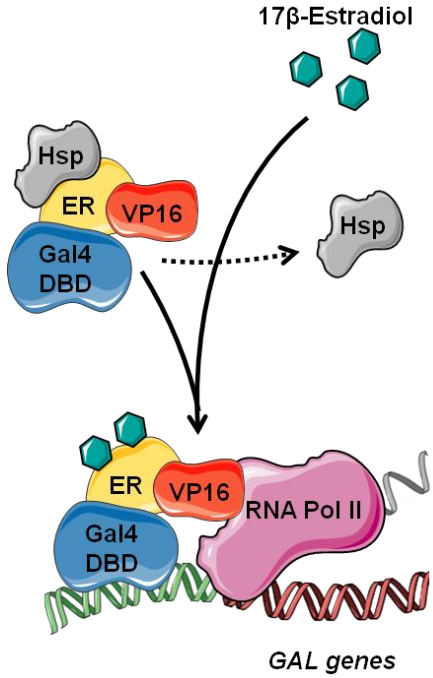

Figure 12. Expression of *GAL* genes with 17β-Estradiol. Gal4DBD-ER-VP16 protein remains in the cytoplasm sequestered by chaperones (Hsp) that binds the ER domain. When 17β-Estradiol is present it binds to the ER domain releasing the construct from the chaperones and allowing it to go to the nucleus. There, Gal4DBD bind to promoter regions of the *GAL* genes and VP16 recruits RNA Pol II to start transcription.

2.3.4 Use of E.coli proteins in yeast

The prokaryote Tet system (Gossen et al., 1992) can be implemented in yeast and mammalian cells to control gene expression upon addition of tetracycline and its derivatives (i.e. doxycycline). This system has two parts: the tetracycline transactivator (tTA) fused to a VP16 protein and the responsive Tet operators (tetO). Under basal conditions, the tTA-VP16 binds to the DNA at the tetO regions and triggers transcription. Presence of tetracycline and its derivatives binds the tTA domain blocking its interaction with the DNA. This is known as the Tet-OFF system (Figure 13A).

Mutations of the tTA domain can generate the opposite effect. Changing four aminoacids of the tTA domain generates the reverse transactivator domain (rtTA). In this scenario rtTA can no longer bind the TetO regions; however, addition of tetracycline and its derivatives complement the rtTA mutations and allow the rtTA-VP16 protein to bind the DNA and trigger transcription (Gossen et al., 1995). This is known as the Tet-ON system (Figure 13B).

Another interesting system from prokaryotes is the LacI repressor. LacI can be expressed in bacteria, yeast and mammalian cells and it binds to the LacI operons (LacO). In bacteria, LacI is constitutively expressed and blocks transcription of genes to use lactose. When lactose is present it binds LacI changing its conformation and making it unable to bind the LacO (Jacob et al., 1961). LacI binding to LacO traps RNA Pol II and prevents transcription (Daber et al., 2007). Addition of LacO to the 3' of a promoter region makes the promoter repressible by LacI (Grilly et al., 2007; Stricker et al., 2008) (Figure 13C).

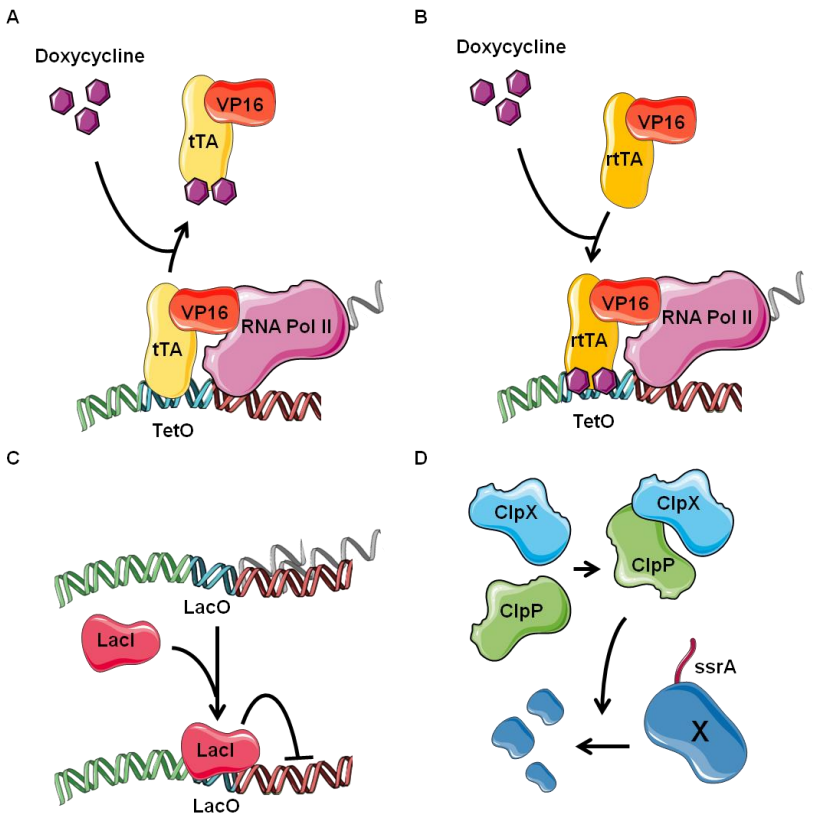

Figure 13. Use of *E.coli* proteins in yeast. (A) Tet-OFF system consists of a tTA-VP16 construct where tTA bind to TetO regions and VP16 recruits RNA Pol II to trigger transcription. Addition of Doxycicline blocks tTA binding to TetO and shuts down transcription. (B) Tet-ON system contains a mutated version of the tTA called rtTA. rtTA-VP16 cannot bind to TetO; however addition of Doxycicline allows rtTA to bind to TetO and VP16 recruits RNA Pol II triggering transcription. (C) LacI repressor can bind to LacO sequestering RNA Pol II and abolishing transcription. (D) ClpX and ClpP protein form a complex known as ClpXP proteasome. ClpXP detects ssrA tagged proteins and degrades them.

The aforementioned systems are useful to regulate transcription, but sometimes there is the need to have a postranslational control. Here, the ClpXP proteasome of bacteria is an interesting system to control protein degradation and can be used in yeast (Gottesman et al., 1998; Karzai et al., 2000; Stricker et al., 2008). ClpXP proteasome is made by two complementary proteins: ClpP and ClpX. When both proteins are present, they form the ClpXP proteasome, which can recognize the ssrA sequence (AANDENYALAA) in a protein and lead to the degradation of the tagged protein (Figure 13D).

2.4 Technologies for synthetic biology

Synthetic biology benefits from advances in some engineering disciplines and the different fields of biology. Initially synthetic biologists faced the problem of engineering biology with a limited set of tools to manipulate DNA. These tools mainly consisted of PCRs to obtain DNA fragments and enzyme-mediated assembly to build bigger constructs. Although these technologies are still in use nowadays, new tools are taking the lead speeding up the process.

For example, in the last decade DNA platforms that can synthesize DNA pieces up to 10 kb have appeared. Thanks to these advances even entire yeast chromosomes have been built from scratch (Richardson et al., 2017; Xie et al., 2017). This ability to synthesize big pieces of DNA allows scientists to build things that were not possible some years ago.

Another boost to synthetic biology came from the generation of libraries with biological parts and new methods for DNA assembly. On one hand the increasing knowledge and

biological parts available has led to the generation of libraries of biological parts with different functions such as: iGEM Registry of Standard Biological Parts (Chen et al., 2013) or the BioBricks™ format that allow easy straightforward assembly. On the other hand, to assemble these parts other methods not based on the classic restriction enzyme cloning has been developed, such as Golden Gate, TOPO, Gibson assembly or Gateway cloning.

Moreover, advances in gene modification and genetic reprogramming, such as the widespread use of the CRISPR/Cas9 (Cong et al., 2013; Mali et al., 2013) and its derivatives, offer the possibility to easily implement many changes in cells and wire logic gates, giving raise to promising results in biological computations (Khakhar et al., 2016; Kiani et al., 2014; Nissim et al., 2014).

The generation of tools to manipulate and engineer cells has made a huge impact in the field causing it to rapidly grow. However, with the increasing number of circuits and its different applications came the need to not only modify cells but their extracellular container. For example, the development of new biocompatible hydrogels (Shao et al., 2017) and cell encapsulation (Xie et al., 2016) has allowed to introduce synthetic modified cells in mice that can regulate glucose homeostasis in a β-cell like manner. Another powerful tool is microfluidic devices (Lin et al., 2012). These devices can finely control the extracellular environment and even isolate individual cells in different compartments (Din et al., 2016). One of the problems with microfluidics is that the process of creating them is manual and prone to failure, however new technologies such as 3D printing may overcome this limitation in a near future (Bhattacharjee et al., 2016).

2.5 Applications of biological computation

We live in a hyper connected world surrounded by computers that we use daily. Nowadays the information flows fast; however, sometimes, is hard to remember that we have not always benefited from these technological advances. The first smartphone appeared 10 years ago, Internet has been used daily for less than 20 years and if we go back to 60 years ago the society we have nowadays could only be glanced by reading sci-fi novels.

Synthetic biology nowadays is at a proof of concept state; however, the ability to manipulate living organisms holds the promise to develop new materials, produce complex biomolecules, revolutionize medicine and replace fossil fuels. In the last decade, hundreds of logic circuits have been built and some examples of interesting applications appear on high impact journals every month. Some devices try to address health problems, others environmental problems, others improvement of biotechnological processes. Just as an example, recently, mammalian cells have been engineered to sense extracellular blood glucose levels and produce insulin to restore glycemic levels in mice, addressing Type 1 Diabetes (Shao et al., 2017; Xie et al., 2016). Other potential applications have been developed to treat disease such as cancer or metabolic syndrome (Bacchus et al., 2013; Wu et al., 2015; Ye et al., 2013; Ye et al., 2011). More examples, include the generation of T-CAR cells, T lymphocytes modified to identify and kill tumor cells (Chen et al., 2010) which are making its way into the clinic; or the generation of antimalarial drugs by synthetic complex circuits (Paddon et al., 2013; Ro et al., 2006). Other devices aim for production of new biomolecules and bioremediation, some focus on generating alternatives to plastic usage (Aldor et al., 2003) or biofuel production (Peralta-Yahya et al., 2012a).

Despite the success in some of the aforementioned applications, we are yet far away to make biology an engineering discipline that allows to reliable engineer and produce on demand living organisms to suit our needs. However the future that synthetic biology envisions is still appealing.

OBJECTIVES

Our group has historically been interested in understanding how eukaryotic cells sense and respond to environmental cues and this has led to a deep understanding of *S.cerevisiae* biology and signaling pathways. Some years ago, we became interested in using this knowledge to redesign signaling pathways to generate new yeast strains that behave in a predictable manner.

The aim of this thesis project is to explore the computation capabilities of multicellular circuits.

Specifically, the main objectives of the PhD thesis are:

1- Use spatial segregation together with multicellular computation to build circuits with low wire requirements.
2- Generate multicellular memory circuits.
3- Build circuits that respond to changing concentrations of extracellular glucose with a tunable dynamic behavior.

RESULTS AND DISCUSSION

Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia

Javier Macia*, Romilde Manzoni*, Nuria Conde*, Arturo Urrios, Eulàlia de Nadal, Ricard Solé, Francesc Posas

PLoS Computational Biology 12.2 (2016): e1004685.

* These authors have contributed equally to this work

-RESULTS-

Macia J, Manzoni R, Conde N, Urrios A, de Nadal E, Solé R, et al. Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia. PLoS Comput Biol. 2016 Feb 1;12(2):e1004685. DOI: 10.1371/journal.pcbi.1004685

Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia

Javier Macia*, Romilde Manzoni*, Nuria Conde*, Arturo Urrios, Eulàlia de Nadal, Ricard Solé, Francesc Posas

PLoS Computational Biology 12.2 (2016): e1004685.

* These authors have contributed equally to this work

-DISCUSSION-

Synthetic biology has successfully engineered biological circuits that are able to perform different functions (Elowitz et al., 2000; Ye et al., 2013; Zhou et al., 2016). One of the challenges in the field is how to build circuits with higher degrees of complexity, which required solving problems such as scalability and reusability of the parts.

The field has focused on developing circuits in single cells by engineering genetic networks (Elowitz et al., 2000; Li et al., 2015). One of the problems of this approach is how to connect the computational modules within a cell. In a circuit, the computation normally is performed by connecting basic logic gates. Inside of a cell, logic gates are normally transcription modules that produce a molecule (wiring) that affects another transcription module. Since logic gates inside of a cell are not physically isolated, as it is the case in electronic circuits, there is the need of specific wires to connect specific logic gates to prevent crosstalk. This means that to build more complex circuits which involve additional wires, different wiring molecules are needed. This requirement introduces complexity since there is a limited number of molecules that can be used as wires, and they have different chemical and dynamic properties as well as there is the possibility that one wiring molecule could interact with another (crosstalk).

To address this issue, it was proposed that circuits could be implemented by the interaction of several different cells within a consortium (Regot et al., 2011; Tamsir et al., 2011b). In this multicellular context, each cell implemented a logic gate and communicated with other cells with a secretable wiring molecule. This scenario reduced the requirements for cell engineering since cells carry only a part of the circuit inside, instead of the whole circuit. Moreover, since cells behave like logic gates, by changing

the combination of cells within a multicellular consortia different circuits can be built, allowing cells to be reused in different circuits.

The use of multicellular consortia was explored together with spatial segregation (Tamsir et al., 2011b) or with distributed output production (Regot et al., 2011). However, this approach still has room for improvement.

In this work, we explored the combination of multicellular consortia and spatial segregation to improve biological computation

Multicellular logic circuits with distributed computation show reduced wiring requirements

With the aim to reduce wires and genetic engineering in multicellular circuits we developed a new architecture that we called ILF (Inverted Logic Formulation) to build circuits. This new architecture addressed two of the key challenges (wiring and cell engineering) to build complex circuits.

Briefly, given that any logic function can be split, we proposed to distribute the computation into a multicellular consortium that can be separated physically in isolated chambers. By exposing all chambers to the same combination of inputs and considering all outputs produced from the chambers, the final output of the computation can be assessed (Fig. 1A).

As a general architecture, a chamber contains a consortium of cells composed by two categories of cells: input layer cells (IL) and output layer cells (OL). Input layer cells respond to a given input and produce a secretable wiring molecule. There are two types of IL cells according to their internal architecture: identity cell (ID), that only produces the wiring molecule when the input is sensed, and NOT cell (NOT) that in basal conditions produces the wiring molecule, but upon addition of the input represses output

production. The second type of cell, OL cell, senses the wiring molecule and displays a NOT logic response producing the output of the computation in the chamber only in the absence of the wiring molecule. IL cells that share the same chamber produce wiring molecule molecules, since the wiring molecule produced by IL cells is the same, the chamber where cells are contained works like an OR logic gate, accumulating the wiring molecules and mixing them independently of the IL cell that has produced it (Fig. 1A). Moreover, each chamber has a different combination of IL cells and thus may generate a different output in response to the same combination of inputs. By implementing an OR gate to compute the outputs of all chambers, it is possible to compute the final output of the circuit (Fig. 1B).

With this method, it is possible to implement a vast number of computations with low requirements in wiring molecules and cell engineering. We reduced the wiring molecules needed to one. The cells that are needed for a circuit (Z) scales linearly with the different number of inputs we want to sense (N), Z = 2N+1 (ID and NOT cells for each input plus an OL cell) and the maximum number of chambers needed (M) increases according to $M = 2^{N-1}$.. Of note the number of different logic functions (B) that can be implemented grows super exponentially B = 2^2^N (Fig. 1C).

Overall, this method shows that by splitting the computation in multicellular consortia and distributing them in different chambers it is possible to build complex circuits that cover hundreds of different functions. Thus, the main advantage of this architecture is that it allows to implement many different complex circuits by engineering few cells with the easiest logic functions possible (ID and NOT), and that these cells can be reused to build different circuits without spending time to reengineer them.

A library of cells with minimal genetic manipulation serves to create *in vivo* multicellular consortia that specifically respond to different inputs

We built a library of engineered *S.cerevisiae* cells to implement the ILF method described before. To demonstrate the *in vivo* feasibility of this approach we created a library of cells that can respond to up to 6 inputs (Fig. 2A). IL cells were engineered to sense extracellular inputs such as doxycycline (DOX), aldosterone (ALD), dexamethasone (DEX), 17β-estradiol (EST), progesterone (PRO) and α-factor pheromone from *Candida albicans* (αCa). This library was constructed by transforming cells with constructs that expressed specific receptors for each input. Basically, there were two types of IL cells for each input; ID cells sensed the input through a receptor that triggered the transcription of a promoter that produced α-factor from *S.cerevisiae* (αSc) which was then secreted to the media. In these circuits αSc worked as the wiring molecule. NOT cells produced αSc under a modified constitutive *TEF1* promoter that was inhibited by the LacI repressor. In those cells, the input triggered the production of LacI which in turn repressed the production of αSc.

There were two main types of OL cells that contained a receptor for αSc which triggered a pathway that lead to the degradation of a fluorescence protein (GFP or Cherry) (OL cells #1 and #2 respectively) or to the inhibition of the production of a secretable molecule (αCa) (OL cell #3).

In addition to IL and OL cells, we developed and additional third type of cell, called buffer cell, that could sense αCa and produced a fluorescence output. The behavior of this cell was step-like and it was useful to generate a digital output when collecting the different αCa produced by different chambers (Fig. 2B). Thus,

this cell was useful to implement the final OR gate when using OL cells #3 in the circuits. All these cells were characterized and exhibited no crosstalk between inputs or deficiencies in cellular growth. (Fig. 2D, Fig. S6-S9)

In our setup, the computation was distributed among different chambers. Each chamber produced an output. The final output of the circuit came from doing an OR gate between the output of OL cells of each chamber. If OL cells #1 or #2 were used, the output was the generation of a fluorescent molecule. Chamber's fluorescence was analyzed individually with flow cytometry. Once the data was acquired we generated a software that received the fluorescence data of the chamber and automatically performed the OR gate yielding the final output of the circuit. While this method was useful for systems that have a biological part (the circuit) and a machine part (the reader and the software) like a biosensor, it cannot be applied to a fully biological context. For this reason, OL cell #3 was developed, which produced a secretable functional molecule. When OL cell #3 was used, the final OR gate was implemented by collecting the supernatants from all the chambers.

We observed that this approach produced a non-digital output in some circuits. This was due because the final output could come from one or more than one chamber, since there are circuits that to a given combination of inputs have more than one chamber producing the output; then, the final production of the output molecule is higher. To correct this phenomenon, we implemented a buffer cell which, as mentioned before, had a step-like behavior and respond to the output produced by OL cell#3 producing the final output (fluorescence) with a digital behavior.

Combinatorial modular organization of a minimal cell library permits the implementation of complex logic circuits

The previously described library of cells was used to build different circuits. We initially built a 3-input majority rule (Fig. 3), that gave an output only when more than 2 inputs were present. This circuit is used in electronics as a safeguard mechanism ensuring that if some minor part of the circuit fails it can continue functioning. This circuit was used as a proof of concept to test the library and its computational capabilities. Moreover, this circuit had the characteristic that for some combinations of inputs more than one chamber was producing the output (Fig. 3). This was extremely useful to build and test the buffer cell to obtain a digital output.

After building a 3-input circuit, we increased the complexity by building a 4-input circuit, a 2-bit comparator (Fig. 4). This circuit was able to respond to 4 inputs. A pair of inputs was used to codify for a number and the circuit compared the two numbers (a, b) giving three possible outputs: a>b, a=b, a<b. By building this circuit, we demonstrated that our approach is not limited to one output production, since in this setup they produced two different fluorescence molecules.

At last, we wanted to test if we could build a 6-input circuit by using all the cells of the library to achieve a complexity that nobody had achieved before. The circuit chosen to implement a 6-input circuit was a multiplexor 4-to-1 (Fig. 5). This circuit sensed four inputs and used two additional inputs as selectors, that selected to which one of the four initial inputs the circuit responded. This was an interesting application to build a multipurpose circuit that can respond to different situations in different ways by changing the combination of the selectors.

All circuits behaved as expected and showed that the ILF architecture using simple engineered cells with one-input logic response together with using space as a computational parameter can be used to build complex circuits.

Perspective

One of the aims of synthetic biology is to build living organisms that behave in a predictable manner and have new biological functions with properties of interest, such as bacteria that generate plastics (Aldor et al., 2003), yeast cells that produce biodiesel (Peralta-Yahya et al., 2012b) or mammalian circuits that can detect tumor formation (Li et al., 2015). At the core of these there are decision making circuits.

There are many ways to build logic circuits. In general, circuits are generated to produce one specific biological function and they are unique and it is difficult to reuse their parts to build new circuits. One of the goals is to achieve a general architecture that allow a flexible, scalable and reusable building of complex logic circuits. In this work, we have demonstrated that by using multicellular consortia together with spatial segregation, complex circuits can be built with minimal genetic engineering. The process of building circuits is straightforward and we have developed a method that transform any given logic function into a combination of cells that need to be cocultured in different chambers, making it easy to build circuits. Additionally, it permits to reuse its parts once the library of cells has been engineered.

This architecture can be further explored to build circuits that can be reprogrammed or contain memory modules. For reprogramming, since chambers contain ID or NOT cells for each input, it is possible to combine this two logic gates into a 2-input

XOR gate that upon addition of a reprogrammer molecule responds with an ID or NOT logic to the input. In this way, the circuit will have XOR cells in all the chambers and the presence of different reprogrammer molecules will change the configuration of ID or NOT cells in each chamber, changing completely the computation of the circuit to any given set of inputs. Reprogramming may be interesting in applications where there is the need of a circuit that can display different logic functions but it may be difficult to physically separate the specific cells in different chambers. In this scenario, it is easier to have a multipurpose circuit that can be reconfigured by the presence of additional chemical molecules.

Memory is an interesting property to implement in logic circuits. It allows circuits to respond, for example, to a combination of inputs and keep responding to them once removed from the media. This may be useful for circuits that require an inductor molecule. If the circuit has memory the inductor can be removed from the media early on, reducing costs but not affecting circuit performance. Additionally, combining memory together with logic circuits can allow for the creation of circuits that respond to a combination of inputs and then change to respond to a second combination of inputs in a different way. These are called sequential circuits and they have different behaviors according to past and present inputs. Implementation of memory in circuits can be achieved by either introducing feedback loops that maintain the levels of wiring molecules even in the absence of inputs, or by changing the state of the cells, by introducing transcriptional switches or by changing their DNA using DNA recombinases.

This work sets the basis for the use of multicellular consortia to build complex logic circuits effortlessly, and it should be understood as a proof of concept. Despite all the promising results

we have shown, it is worth to notice that this method has no clear benefits when building simple circuits that respond to one or two inputs and on those that the genetic engineering required is more difficult than by using other more straightforward approaches. Also, it is important to stress that the use of multicellular consortia has benefits: the circuit complexity can be split in different cell types reducing cell engineering and the metabolic burden of the cell, and allows to reuse cells to build different circuits. However, it also has clear disadvantages such as population control and the maintenance of population proportions of different cell types within the consortia. Since circuits use an external wiring molecule by using multicellular consortia, the levels of the wiring molecule change by the activation of the cells but also by the number of cells. This means that even if a cell is not active but has a little bit of basal activity, it may alter circuit performance. Furthermore, if one strain displays growth defects, it will eventually disappear from the multicellular consortia, due to competition with other cells, which will lead to changes in the circuit behavior. Moreover, in some cases, the problem is not the ability to respond to several inputs but the proper dynamics of the response. These are questions to be addressed in further studies.

Personal contribution

My personal contribution in this study was to engineer cells (both IL and OL cells and their characterization). I also performed some supplementary experiments, and I participated in the experimental design, data analysis and writing of the manuscript.

A Synthetic Multicellular Memory Device

Arturo Urrios*, Javier Macia*, Romilde Manzoni*, Nuria Conde, Adriano Bonforti, Eulàlia de Nadal, Francesc Posas and Ricard Solé

ACS Synthetic Biology 5.8 (2016): 862-873.

* These authors have contributed equally to this work

-RESULTS-

Urrios A, Macia J, Manzoni R, Conde N, Bonforti A, de Nadal E, et al. A Synthetic Multicellular Memory Device. ACS Synth Biol. 2016 Aug 19;5(8):862–73. DOI: 10.1021/acssynbio.5b00252

A Synthetic Multicellular Memory Device

Arturo Urrios*, Javier Macia*, Romilde Manzoni*, Nuria Conde, Adriano Bonforti, Eulàlia de Nadal, Francesc Posas and Ricard Solé

ACS Synthetic Biology 5.8 (2016): 862-873.

* These authors have contributed equally to this work

-**DISCUSSION**-

Memory is an interesting feature and key component of many biological processes that allow cells to remember previous inputs (Jacob et al., 1961). Several synthetic memory devices were built inspired by transcriptional networks in bacteria that can store information (Inniss et al., 2013). Stablishing circuits with memory may have benefits in industrial applications or for developing more complex circuits such as sequential circuits.

Memory can be established in different ways. In an early work, some simple memory devices were developed in *E.coli*. The simplest memory device reported there held 1 bit of memory (one binary state or piece of information, that can be either 1 or 0). This was done by engineering two mutual repressors yielding a double negative feedback loop (Gardner et al., 2000). Memory has also been established with only one component by establishing a positive feedback loop in yeast (Ajo-Franklin et al., 2007). Recently other approaches involving the use of specific DNA recombinases have been used to establish memory at the DNA level (Siuti et al., 2013).

However, memory has not been studied in a multicellular consortium. We reasoned that if a double negative feedback loop can establish memory in a transcriptional network, it should also establish memory in a multicellular consortium with two cell populations that inhibit each other.

In this work, we built a multicellular memory device that can store 1-bit of memory.

Design of a Multicellular Memory Device

Among the different configurations that can serve to establish memory in a multicellular device, we choose a 1-bit memory device known as flip-flop. The flip-flop can be established

by two NOT cells that inhibit each other (Fig. 1) and is a fairly robust architecture easy to connect with other cells, as was pointed out in previous theoretical studies (Sardanyés et al., 2015). Of note, we had experience building NOT cells, and some of the cells used in this circuit were already built and used in other previous circuits (Macia et al., 2016).

Two-Cell Implementation of a Biological Memory Device

To implement a multicellular device, we engineered two yeast cells populations that inhibited each other by producing specific pheromones. One cell (NOTCa) produced and secreted α-factor from *S.cerevisiae* (αSc) with a modified *TEF1* promoter that was inhibited by the LacI repressor. This cell sensed α-factor from *C.albicans* (αCa) which triggered the expression of LacI, repressing the production of αSc. The other cell (NOTSc) worked in the opposite way, it sensed αSc and repressed the production of αCa (Fig. 2b). To check cell performances, we measured αSc or αCa produced by adding a third type of cell that responded specifically to each of the pheromones and produced a fluorescent protein (Fig. 2c).

We observed that both cells produced their respective pheromone (Fig. 3a), and that both pheromones were sensed separately and there was no crosstalk between pheromones and receptors (Fig. S2). Furthermore, the production of the pheromone of one cell was repressed by the presence of the pheromone produced by the other cell (Fig. 3a), also named as counter-pheromone from here on. Additionally, we found that 1-hour incubation in the presence of the counter-pheromone was enough to inhibit pheromone production, a repression that was fully recovered after 16h of removal of the counter-pheromone (Fig. 3b).

The Multicellular Memory Device Triggers a Bistable Dynamic Response

To test if a 1-bit memory device can efficiently be established in a multicellular consortium, we mixed NOTCa and NOTSc cells. We externally added αSc or αCa to the consortium for 4 hours, removed the media and measured the production of αSc or αCa of the consortium by incubating the supernatant with cells that sensed and distinguished the pheromones and produced a fluorescence output. We observed that the initial incubation with αSc inhibited αCa production even after 48 hours of adding the initial input, while the initial incubation with αCa maintained the production of αCa levels high even after 48 hours. This indicated that the consortium established a memory state (Fig. 4).

First, this was confirmed by the fact that, the initial input was removed to the media so it was not its presence what we were measuring after 4 hours. And second, cells were fully recovered from their initial repression after 16 hours, so if the repression was still present at 48 hours it must be due to the production of the counter-pheromone by the other cell, and hence establishing the memory. Correspondingly, the circuit worked in both directions since it displayed an opposite but equivalent behavior when it was initially incubated with either synthetic αSc or αCa.

We also tried to measure the levels of αSc as we did for αCa but we did not detect any signal (Fig. S9). Therefore, we modified NOTSc to include a direct fluorescent readout of the activity of the cell (Fig. S10). This was done by adding a plasmid that contained a pheromone inducible promoter and an unstable short live GFP version (NOTSc GFP*). Using this approach, we could effectively measure αSc with a direct method (Fig. 7). This suggest that αSc may be sequestered at the cell membrane or

plastic walls of the reservoir and thus an indirect measurement was not suitable for quantifying its presence.

We conclude that NOTCa and NOTSc cells when mixed together form a multicellular consortium with memory capabilities to remember past inputs.

Memory State Changes Can Be Externally Triggered

If a memory state is stablished we reasoned and modelled that we should be able to change between the states by either externally raising the levels of the counter-pheromone or by reducing the levels of the pheromone by adding a specific protease (Fig. 5).

To test this possibility *in vivo*, the consortium was exposed to either αSc or αCa for 4 hours and memory state was assessed after 16 hours. Then, after 16 hours the consortium was exposed to the counter-pheromone or to a specific protease that degrades the pheromone, and the memory state was checked again after 16 hours (Fig. 6). As predicted by the model , the circuit changed states when adding the counter-pheromone or by removing the external pheromone by adding the specific protease.

From these results, we conclude that the memory established in the consortium is an extracellular parameter driven by the levels of pheromone or counter-pheromone. Alteration of these levels by either addition or depletion of the pheromones affects the memory state.

Implementation of a Memory Device in a Microfluidic Platform

Establishing a memory device in a multicellular consortium allowed to modify the memory as an extracellular parameter that can be altered by protease or pheromone addition. To better study

how the circuit responds, we grew the multicellular consortium in a microfluidic chamber that was placed under a microscope. To follow the memory circuit performance in this setup, we used NOTCa cells together with NOTSc GFP* cells that produce a fluorescence output. We observed that the addition of αSc for 3 hours was enough to maintain a memory state for up to 8 hours. After establishing the memory, we added the ScBar1 protease or αCa and we observed the change in the memory state. Additionally, we observed that increasing the flow in the microfluidic chamber also caused changes in the memory state, suggesting again that memory acted as an extracellular parameter that could be externally controlled (Fig. 7).

Perspective

This work demonstrated that it is possible to establish a 1-bit memory device in a multicellular consortium. From the engineering point of view our method involved cells that were easy to engineer with a one input NOT logic gate. This system also is modular and the addition of new cell types can affect the levels of the pheromones. Thus, memory devices able to respond to a broad range of different inputs can be easily generated without reengineering the core memory module. In addition to these advantages in cell engineering and modularization, this system also allows to tune the memory state by altering the extracellular media without affecting gene expression.

Despite these benefits, this system requires an active maintenance of the memory state by the constant production and secretion of pheromones. Moreover, perturbations that unbalance cell proportions within a population can modify the memory state, making the system to malfunction. Alternative methods to maintain

memory involves writing the memory at the DNA level. In this scenario, a genetic construct can change upon addition of an input that leads to a DNA recombinase to be activated and flip the genetic construct making it active or inactive. Such systems have already been implemented to build genetic DNA memory (Bonnet et al., 2013b; Siuti et al., 2013) but further work and characterization on how DNA recombinases work needs to be performed to be used effectively.

Personal contribution

My personal contribution in this study was the engineering of the cells. I did experiments and data analysis and I participated in writing the manuscript. The theoretical framework and models were developed by Dr. Javier Macia.

Plug-and-Play Multicellular Circuits with Time-Dependent Dynamic Responses

Arturo Urrios*, Eva Gonzalez-Flo*, David Canadell, Eulàlia de Nadal, Javier Macia[ǂ] and Francesc Posas[ǂ]

Submitted

* These authors have contributed equally to this work

[ǂ] Corresponding authors

-RESULTS-

Urrios A, Gonzalez-Flo E, Canadell D, de Nadal E, Macia J, Posas F. Plug-and-Play Multicellular Circuits with Time-Dependent Dynamic Responses. ACS Synth Biol. 2018 Apr 20;7(4):1095–104. DOI: 10.1021/acssynbio.7b00463

Plug-and-Play Multicellular Circuits with Time-Dependent Dynamic Responses

Arturo Urrios*, Eva Gonzalez-Flo*, David Canadell, Eulàlia de Nadal, Javier Macia[ł] and Francesc Posas[ł]

Submitted

* These authors have contributed equally to this work

[ł] Corresponding authors

-DISCUSSION-

One of the most relevant biomedical problems is Diabetes type I which affects around 40 million people all around the globe (Shaw et al., 2010). Synthetic biology holds the promise to generate biological circuits to solve several biomedical problems. Although some circuits are easy to build, building bigger complex circuits for specific situations it is still an elusive nontrivial problem.

Synthetic biology proposes to generate synthetic circuits able to produce insulin in response to glucose to emulate the function of a pancreatic beta cell in another cell type. Doing this has two clear advantages. On one hand, using another cell type with a different circuit architecture ensure that the mechanism that kills pancreatic beta cells does not act in the engineered cells. On the other hand, by building *de novo* a glucose regulated synthetic circuit, several components can be tuned to generate new behaviors that work better in a type I diabetic patient. Several approaches have been done in this area, the most promising one is a synthetic gene circuit that produces insulin in response to extracellular glucose. The circuit was engineered in HEK293T cells. Cells were encapsulated and implanted into diabetic mice reducing the diabetic phenotype (Xie et al., 2016), however there is still room for improvement. For example, the circuit was fairly slow and with a simple input-ouput dynamic production that may not be suitable for long-term treatment

In this study, we proposed to build a multicellular circuit able to sense extracellular glucose levels and produce a response mimicking the function of a pancreas. This circuit was developed in yeast cells since they are easy to manipulate and can quickly respond to a broad range of extracellular glucose levels.

In this setup, the multicellular consortia responded to different levels of extracellular glucose and produced a biological

response, i.e. secretion of insulin or glucagon. The computation in the device was split in three parts: a sensor module, a modulator module and an effector module. By having the circuit split in different cells, genetic manipulation and metabolic burden of the cells were reduced. Additionally, splitting the circuit in different modules of cells allowed to study and characterize cells independently and then, couple them to make the final circuit.

This synthetic circuit was created to get insights on the circuitry and architecture of a glucose regulated circuit. Additionally, the knowledge gained can be further optimized in mammalian cells to explore its use in a diabetic animal model.

Sensor cells are induced by extracellular glucose levels

Glucose is the primary carbon source for *S.cerevisiae* and many other organisms. While multicellular organisms have evolved mechanism to ensure stable levels of extracellular glucose, unicellular organism adapt to available extracellular glucose. To do that, yeast have developed mechanisms to quickly adapt and sense glucose availability and optimize its uptake.

Yeast cells can sense environmental glucose levels and adjust the expression of different hexose transporters to maximize glucose uptake. Several studies pointed out that transcription of some hexose transporters was dependent on extracellular glucose levels (Özcan et al., 1999). We tested how glucose affected the transcription of several HXT promoters (P_{HXT1}, P_{HXT2}, P_{HXT3}, P_{HXT4} and P_{HXT7}) by building constructs that expressed *S.cerevisiae* α-factor (αSc) under the control of these promoters. The constructs were transformed into yeast cells.

We exposed the engineered cells to different extracellular glucose levels (low = 0,5%, mid = 2%, high = 5%) and measured

the αSc in the media by adding one cell that detected extracellular αSc and produced a fluorescent protein (i.e. GFP). We found that cells containing constructs with promoters $P_{HXT2,}$ P_{HXT4} or P_{HXT7} repressed αSc secretion at high of glucose. P_{HXT7} showed the greatest difference between being active at 0.5% of glucose and repressed at 2% or 5% of glucose. On the other hand, cells containing constructs with promoter P_{HXT1} secreted αSc at high levels of glucose (>2%) (Fig. 1a). P_{HXT1} and P_{HXT7} were selected as potential sensors for high and low extracellular glucose respectively. We built sensor cells (HXT7::αSc and HXT1::αSc) with two copies of each construct to make them more efficient in αSc production. These sensor cells were incubated with different levels of extracellular glucose (0,1-0,5-1-2-3-5%) and αSc was measured. HXT7::αSc cells secreted αSc when extracellular glucose was <2% while HXT1::αSc cells secreted αSc when glucose was >2% (Fig. 1b). Moreover, when cells were shifted from media containing specific glucose levels to media with different glucose levels, they did not display a strong memory and modified their response to the present extracellular glucose concentration (Fig. 1c).

Therefore, these sensor cells are useful to transform extracellular glucose levels into αSc signal that can be further processed. In this way, we developed and serve as two glucose sensors with an opposite behavior and a threshold of activation at 2% of glucose.

Effector cells responded to αSc by producing insulin or glucagon.

Yeast cells were modified to sense αSc and produce different outputs. They produced GFP under a pheromone inducible promoter (P_{FUS1}) which was used to test the circuit

behavior easily (reporter cells) (αSc::GFP cells) or a secretable molecule, i.e. insulin or glucagon (effector cells) (Fig. S1). αSc::INS cells were transformed with P_{FUS1}-αINS, a construct that express a modified version of an insulin analog precursors (IAPs) with a short C-chain (EWK) fused to the preproleader sequence of α-factor for efficient secretion in yeast under a pheromone inducible promoter (Kjeldsen T *et al*., 2002). αSc::GCG cells were transformed with P_{FUS1}-αGCG that expressed a modified version of glucagon fused to the preproleader sequence of α-factor for efficient secretion in yeast under a pheromone inducible promoter (Moody AJ *et al*., 1987). Both effector cells were incubated with different amounts of αSc and insulin or glucagon presence were quantified in the supernatant by using specific Mercodia ELISA kits (Fig. S1b,c). Both effector cells showed a similar behavior, however αSc::INS cells were more efficient than αSc::GCG mainly because the construct was optimized for yeast secretion. These results suggested that αSc can be transformed into many different biological outputs by changing the cell configuration content in the effector cell layer.

In order to regulate the production of the hormones by extracellular glucose levels we mixed sensor and effector cells. HXT1::αSc cells were coupled with αSc::INS cells, we found that production of insulin occurred only at high levels of glucose with a transfer function that resembled the behavior of HXT1::αSc cells. On the other hand, HXT7::αSc cells were coupled with αSc::GCG cells. We found that the production of glucagon occurred only at low levels of glucose with a transfer function similar to HXT7::αSc cells (Fig. 2).

One concern when using multicellular circuits is that the signal to output production might highly delayed because of the

delays involves in production and secretion of the wiring molecule. Here, we studied the kinetic of the responses of both circuits (HXT1-αSc-INS and HXT7-αSc-GCG) and both insulin and glucagon were detected as early as 30 minutes (Fig. S2). Moreover, both circuits were turned on and turned off by changing the glucose in the media and they did not display a strong memory effect. Only a slight leakiness of the HXT1-αSc-INS circuit was observed but was completely shut down after 30 minutes (Fig. S2). Since both layers when analyzed separately did not have a strong memory, we didn't expect a strong memory of the circuit as it is the case.

Modulating input sensitivity in multicellular circuits

Glucose affinity and sensitivity of these devices is mainly determined by the wild type *HXT* promoters involved in each circuit. Tuning the glucose affinity and sensitivity could be extremely useful to use these circuits for biomedical applications. In general, fine-tuning is performed by genetically modifying the wild type promoters and changing their characteristics. By contrast, in a multicellular approach tuning could be achieved by modulating the total amount of extracellular wiring molecule, i.e. αSc.

Since the circuit was split in two layers of cells (sensor and effector) we checked the effect of changing cell proportions between these layers. To study this situation, we used HXT1-αSc-INS circuit. We observed that increasing HXT1::αSc population resulted in insulin production at lower levels of glucose, shifting slightly the threshold of activation (Fig. 3a). This indicates that cell proportions may play a role on modulating circuit sensitivity to extracellular glucose as was further analyzed by means of a mathematical model (Fig 3b,c).

Pulsatile dynamics in multicellular circuits

The activity of these circuits was linked to the levels of extracellular wiring molecule and alteration of those levels modified circuit performance. While previous circuits formed by sensor and effectors cells did not produce time-dependent responses, we hypothesized that by adding a third player to unbalance the wiring molecule levels, we could mimic a pulsatile behavior This was achieved by generating two new cell types, HXT1:αCa and αCa:: Bar1^{Sc}. HXT1:αCa cells produced *Candida albicans* (αCa) at high glucose levels by expressing the corresponding gene under the control of *HXT1* promoter On the other hand, αCa:: Bar1^{Sc} in response to αCa secreted Bar1^{Sc} a protease that specifically degrades αSc (Figure S4,S5).

HXT1:αCa, HXT1:αSc, αSc::INS and αCa:: Bar1^{Sc} were mixed together. In this configuration, glucose signal triggered both and activation and inhibitory response to insulin production. High glucose concentration triggered the secretion of αSc and αCa simultaneously. αSc directly induced the synthesis of insulin while αCa directly induced the secretion of Bar1^{Sc}, degrading αSc (Figure 4). Here, concentrations of αCa:: Bar1^{Sc} cells, the modulator, played a critical role. If the concentration was too low there was no pulse formation, if the concentration is too high there was no output production. Intermediate levels, however, were able to produce a time-dependent response, a pulse (Figure 4c). Experiments were performed during 540 minutes in a semi-continuous setup. Every time cell doubled the culture was diluted by half with fresh media. Insulin levels were measured after culture dilution.

All these results suggest that it is possible to build a multicellular circuit that respond to glucose orl other indicators and produce a biological output with different dynamic behaviors.

Perspective

Glucose is an important molecule to supply energy to cells and sustain several cellular processes. It is the preferred carbon source for many organisms from simple unicellular eukaryotic organisms like yeast cells to more complex organisms like humans. In fact, problems in glucose regulation as it happens in Diabetes lead to many health problems and metabolic disorders that might resultin death if not treated properly.

Over 400 million people are affected by Diabetes (Shaw et al., 2010). Around 10% of them are Type 1 with a defective production of insulin, while the rest are Type 2 and have developed a resistance to insulin. The current treatment for Type 1 Diabetes involves monitoring blood glucose levels and control their levels by daily injections of exogenous insulin. However, several alternative treatments are under development. Some of these approaches involved donor pancreas transplantation (Qi et al., 2014) or *in vivo* differentiation of stem cells to produce functional beta cell (Pagliuca et al., 2014). These approaches may restore insulin production and regulation in the body but are sensitive to be rejected again by the same mechanisms that lead to beta cell deficiency. Other approaches focus in developing artificial pancreas composed by a blood sugar sensor and an insulin pump (Peyser et al., 2014; Russell, 2015). Nowadays these systems have some drawbacks like the storage of insulin and glucagon and the problems associated to having an external device attached to the patient body.

All these approaches have some potential benefits as well as limitations. Advancements in synthetic biology has led researchers to build genetic circuits that produce insulin in response to different levels of extracellular glucose in non-pancreatic cells (Xie et al., 2016). Thus, cells containing a synthetic gene circuit to produce insulin was engineered in HEK293T cells, cells were encapsulated and implanted into diabetic mice. Diabetic mice with the synthetic circuit were able to effectively control its glycemic levels for several days. Some limitations of this study were that cells did not really respond to direct changes in extracellular glucose but to an associated membrane depolarization, which may render the circuit sensitive to non-related changes in calcium levels for instance. Additionally, the circuit was fairly slow and with a simple input-ouput dynamic production. This may be sufficient to control glycemia in mice, an organism that tolerates well both extremely high and low insulin levels, but raises concerns if a direct human application might have the same results.

In our work, we have developed a multicellular circuit that was able to sense extracellular glucose and produce insulin or glucagon. Our circuit was modular and could be easily configured to produce insulin or glucagon with different behaviors which could be tuned by adding additional cells. Moreover, the circuits responded in 30 minutes and produced detectable amounts of insulin or glucagon. However, our circuit have some important drawbacks. I) they responded to glucose levels that were far above the levels of high glucose in humans, ii) the insulin and glucagon produced need further postprocessing to be fully functional, iii) multicellular circuits were sensitive to changes in cellular proportion, iv) yeast as an organism may not be suitable to be encapsulated or implanted in animal models.

These drawbacks are more than sufficient to not use these circuits in an animal models even if we succeed in tuning the sensor cells to work on different glucose levels. We believe that in the future of our approach can be build this circuit into a mammalian cell model. In this scenario, a complex internal architecture and the use of approaches like the ILF method is not required (Macia et al., 2016), However, it is worth investing resources in looking for glucose responsive promoters specially those with a fast activation response. As in yeast, there may be problems maintaining cell populations or cellular proportions. In mammals there are cell types that have cell-cell contact inhibition and this drawback could be solved. Moreover, the problems of producing and efficiently processing insulin in yeast have been already solved for mammalian systems (Hay & Docherty, 2003).

When developing a synthetic circuit to address a biomedical problem it is equally important the cell as the extracellular context, the whole organism. In this sense, we believe that a linear relation between input and output may not be suitable to keep the blood glucose levels in a normal range. However, a time-dependent relation, like a pulse, should be a better approach to maintain blood glucose levels in a normal range. In this case it is important to consider the extracellular context and prioritize building a circuit that takes it into account. Instead, building a synthetic circuit with a reduced cell engineering without considering the dynamic of the response, at the end, may not be suitable to solve the situation. While building time-dependent circuits in single cells may be a challenge, with a multicellular approach, we can change a circuit from being time-independent to time-dependent without modifying the cells, only by adding an additional cell type to the consortia.

Personal contribution

My personal contribution in this study was the engineering of cells used in the study. I did the experimental part and data analysis as well as I participated in writing the manuscript. The theoretical framework and models were developed by Dr. Javier Macia and Eva Gonzalez-Flo.

GLOBAL DISCUSSION

Synthetic biology aims to rationally engineer living organisms (Endy, 2005) in order to produce new biological compounds (Ro et al., 2006), make cellular computers that can solve biomedical problems (Xie et al., 2016) or just to learn how a system works and understand the logic behind it. Synthetic biology benefits from advancements in close related disciplines such as Molecular Biology, Biotechnology or Systems Biology. Sometimes it is hard to set a border of what is Synthetic Biology and what is Biotechnology for instance. However, what makes Synthetic Biology unique is the purpose to rational design or engineer living organism. In practice, Synthetic Biology involves combining computational tools to predict and model how a circuit should behave by building circuits with molecular biology tools and by assembling pre-characterized modules.

While the goal is clear, there is not much consensus about how to achieve flexible, programable, scalable and predictable engineered constructs able to perform complex computations. For example, which biological chassis should be used to contain a synthetic biological circuit? While some researchers choose bacteria for their circuits others use yeast or mammalian cell lines. For instance, to generate a circuit that detects and kill cancer cells, a priori it is easier to generate a circuit in mammalian T-cell than in bacteria, mainly because mammalian cells have evolved additional elements that might be useful to implement a given function and might need fewer engineering steps (Wu et al., 2015). However, for other type of circuits bacteria could be the preferred choice since they are easier to manipulate and display faster growth rates. For the building of our multicellular synthetic circuits we used yeast cells, since we have extensive experience working with this type of cells and it is easy to perform cell engineering. Moreover, yeast life

cycle has two haploid forms that communicate through secretable pheromones. With little engineering, this natural system was transformed into a wiring molecule for our multicellular circuits.

Nowadays, circuits are made by assembling pre-characterized modules and the function of these modules may vary when implemented in different organisms. This should not be a limitation if we understand the molecular biology at the DNA level to predict how a DNA sequence will generate a protein when specific conditions are met and how this protein will fold and interact with other molecules to produce a specific function. However, we are still far from achieving that level of knowledge.

Going back to the goal of generating flexible, programable, scalable and predicable engineered constructs the current tendency in the field involves building circuits inside single cells. Although this approach has led to implement many biological circuits (Furukawa et al., 2015; Li et al., 2015; Tabor et al., 2009; Ye et al., 2011) it faces some limitations in terms of scalability and flexibility.

The main problem is that integrating more complex functions in biological circuits often involves the wiring of several modules. Inside of the cell wiring molecules are all mixed together. Thus, in order to connect different modules, there is the need to use different wiring molecules that are independent of each other and do not show crosstalk or additive effects. Increasing the number of modules to connect, involves an increase in the number of wiring molecules. This fact enhances slight differences among wiring molecules or undesirable effects that can alter circuits function. Additionally, complex circuits consume more cellular resources. These circuits are implemented within a living organism which its main goal is to grow and adapt. If the new synthetic construct is too

demanding, this will affect cell fitness and it will be sensitive to negative selective pressure. Another limitation of this type of approach is that modules cannot be removed or added easily and requires to re-engineer cells when a different logic is required, limiting the flexibility of this method.

In order to overcome these potential limitations, we explored the use of multicellular consortia to build biological circuits. In a previous study, it was demonstrated that by modifying yeast cells to produce and sense pheromones as wiring molecules in response to certain extracellular stimuli it is possible to establish multicellular synthetic circuits (Regot et al., 2011). This approach allowed to build LEGO-like multicellular systems where each cell worked as a module of the computation. This method is flexible because just by exchanging cells that are growing together, different circuits can be implemented without additional cellular engineering. Also, it was shown that this setup allows to implement a distributed output (more than one cell of the consortia gives the final output) which can reduce wiring requirements.

However, that study did not specifically address the optimization of engineering complex multicellular circuits. This was explored in the work presented here entitled *Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia*. We built multicellular circuits combining distributed computation together with spatial segregation and keeping three objectives in mind: reduce cell engineering, reduce wiring requirements and develop a straightforward universal method to build biological circuits (Macia et al., 2016). The implementation of this design fulfilled the three objectives and demonstrated that it is possible to build scalable digital biological circuits by using yeast cells with the simplest logic function possible

(ID or NOT) and communicating them with one wiring molecule. In order to reduce cell engineering, two elements were required, the output of the circuit had an inverted logic and the use of space as a new computational parameter.

While working on the implementation of the design, we found three major limitations for our design, biological, technical and theoretical problems. Our system relies on having different yeast strains growing together. Some yeast cells were modified to sense external inputs and produce or not a wiring molecule. Other cells were modified to sense the wiring molecule and produce or not a desired output. This setup is sensitive to input levels, cell proportions, cell amounts and the level of wiring molecules. One intrinsic limitation is that yeast cells duplicate every 90 minutes. In closed environments, wiring molecules accumulate and since cells divide and keep producing wiring molecules, these accumulate fast. While this is not a limitation to implement a one-time digital response, it can be an issue when building circuits that change their behavior along the time. Also, since we are working in a biological system, there is always a basal activity, while this effect may not be an issue in short periods of time, it is enhanced over time altering circuit's function. For these reasons, keeping cell growth in check is a key point to ensure the long-term stability of these circuits. In fact, this is an issue for general synthetic circuits and not only for multicellular circuits. Of note, multicellular circuits are especially sensitive to cell growth because wiring is extracellular and it is proportional with cell number, thus differences in the cell growth of the different strains configuring the consortia may change their proportions making the circuit to malfunction. Possible solutions to this issue are: produce a cell cycle arrest, reduce cell growth without killing the cells or affecting its

metabolism, change the organisms to other with slower cell growth or use a chemostat to keep cell number stable by washing away cells.

Another limitation of the current setup is that the wiring molecule used was a natural extracellular communication system of yeast (the pheromone pathway). This has two potential limitations, on one hand it may be difficult to implement this system in other organisms, for example, it is not clear whether mammalian cells can produce yeast pheromones effectively. On the other hand, pheromones trigger a cellular response in yeast cells in addition of controlling the synthetic circuit, such as G1 arrest, that can be compensated by making other modifications in the cell (McKinney et al., 1995). Ideally the wiring molecule should only affect the circuit and not interact with other pathways of the cell, for example, this can be achieved by using auxin to communicate cells instead of yeast pheromones (Khakhar et al., 2016)

In the present embodiment, the technical problem is how to isolate cells in individual chambers while collecting the outputs together. If the output of the chambers can be quantified independently, the chambers can be independent tubes where there is no communication between them. The outputs of each chamber can be quantified separately and the OR function can be implemented using a computer. While this approach is effective, it raises some concerns to whether the whole circuit performs the logic function *per se* or whether it needs the aid of a computer to implement the last OR function among chambers. This limitation can be overcome if the output of each chamber is a secretable molecule that can be collected in another chamber. A fluidic system can keep cells isolated and allows to collect the outputs together. Ideally this system might be a microfluidic system with valves. The

main problem is that microfluidic devices are hard to build and the development of a functional prototype can last years. Additionally, the current techniques for production (PDMS molding and bonding) are impractical and prompt to assembly failures. One alternative for rapid prototyping of a functional device is the use of 3D printing technologies (Bhattacharjee et al., 2016). We explored several configurations of resins and the use of stereolithography to generate transparent biocompatible devices (Urrios, Parra-Cabrera, et al., 2016). While this technique allows to easily build devices, the resolution is an issue since channels are around $1mm^2$ cross section. Yeast haploid cells measure around 4 microns, with such big channels cells are not retained in the chambers and are washed away. However, fluidic automation is possible (Au et al., 2015). On top of that, the material is no gas permeable and yeast growing in channels generate gas that lead to bubble formation and distort the flow of the channel. To overcome these limitations, we are currently working on a 3D printed open microfluidic device and studying the use of filters as barriers to keep cells isolated. It is to our believe that by having a reliable platform that allows to spatially segregate cells and modulate the extracellular environment, we can further explore the limits of multicellular computation.

The theoretical problem is that while the design is useful for building complex digital circuits, it has no clear benefits for the implementation of circuits with less than 3 inputs. Therefore, in this scenario, instead of applying this architecture it would be more beneficial to invest in optimizing the dynamics of the parts for a better performance of the circuit. Nowadays, there are no many examples were a circuit with more than 3 inputs is required.

Initially, we explored the use of multicellular consortia to build digital circuits where the flow of information goes in one direction

from input to output. In the work entitled *A Synthetic Multicellular Memory Device* we explored what happens if we allow the information to flow in both directions. We built two yeast strains that are able to establish an extracellular double negative feedback loop (Urrios, Macia, et al., 2016). When one cell was activated by an external input it repressed the other even when the external input was removed from the media. This system was able to maintain a memory state stable up to 48 hours. Albeit this is a very interesting feature, the circuit faced the same problems of multicellular circuits mentioned before. Additionally, it involved active transcription and production of wiring molecules and repressors which can cause metabolic burden to cells in contrast with other methods to build synthetic memories such as DNA recombinases (Farzadfard et al., 2014) that works by changing the orientation of a DNA construct under specific conditions triggering or shutting down its expression. The strength of this design consists on its flexibility. By adding other cells to the circuit, it is possible to implement a memory device able to sense several environmental stimuli without further engineering the cells. Moreover, since the memory is an extracellular parameter, it can be easily tuned without affecting the cells, for example by removing the media or by producing specific proteases.

Along the same lines, we generated a multicellular circuit that did not work with a digital response and displayed a complex dynamic behavior. In the work entitled *Plug-and-Play Multicellular Circuits with Time-Dependent Dynamic Responses,* we explored how the proportion of each cell in the consortia and the configuration of the different cells modulate the response of a circuit to extracellular glucose levels. While there are circuits implemented in mammalian cells that are useful to regulate

glycaemia in diabetic mice (Xie et al., 2016; Ye et al., 2011) they do not directly respond to glucose, their response is slow and they display simple input to output dynamic response. In our work, we explored how to build a circuit with a fast response, that respond to glucose with a modular architecture that make it easy to modify the dynamics. This circuit allowed to study how different levels of cells within the consortia alter the output responses, threshold of activation and how addition of new cells can modify the dynamics of output secretion. While this work used non-physiological levels of glucose and cannot be directly used to treat diabetes in a mouse model, it served to understand how to build a circuit in mammalian cells.

All the studies developed during this PhD served to understand how to implement multicellular circuits and which are the limitations of these type of circuits and the potential applications of this technology. Multicellular circuits have the potential to achieve complex behaviors that a single organism cannot display. So far, we were able to build complex digital circuits, circuits with feedbacks that display memory and circuits that are able to respond to changes in extracellular glucose levels. However, there are many caveats left to address in the future. For example, in terms of flexibility these circuits can be improved by making them reprogrammable. This means that instead of using different cells in a circuit, there would be a type of cell that could display different logics just by the addition of an external molecule. This can be interesting for building multifunctional circuits for environments with a limited external intervention. Another element to explore is space as a computational parameter. There is still room for testing designs and building microfluidic devices with 3D printing. If space

becomes a reliable computation parameter, we can further scale up our circuits to levels that cannot be done in single cells.

In any case, the development of synthetic multicellular circuits is a field with plenty of room for study that may lead to interesting findings and applications in the near future.

CONCLUSIONS

This section provides the conclusions from the different studies performed during my PhD.

Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia

1- With the proper design and architecture, the use of cells able to respond with a simple logic can yield complex logic functions (ILF).

2- Spatially distributing computation reduces cell engineering.

3- Reduction in cell engineering is translated into a higher complexity of the fluidic devices that contain the multicellular circuit.

A Synthetic Multicellular Memory Device

1- Distributing the computation in several cells simplifies the different modules of computation and allows to combine different cells to build complex circuits.

2- When working with multicellular synthetic circuits, the extracellular computation can be as important as the intracellular.

3- Short-term memory is an intracellular property but long-term memory is an extracellular parameter that can be easily tuned.

Plug-and-Play Multicellular Circuits with Time-Dependent Dynamic Responses

1- ILF is useful for building complex digital circuits but for circuits with less than 3 inputs it has no clear benefits.

2- In multicellular consortia, cell proportions are a critical parameter that can change completely the threshold of activation and dynamics of the output secretion of the circuit.

3- When building a circuit for biotechnological application, it is interesting to test the architecture on a model organism to monitor is feasibility.

4- The use of model organisms has limitations due to differences within organisms and the results obtained may be non-direct transferable.

5- The potential of synthetic biology for biomedical or biotechnological applications has no end in sight.

SUPPLEMENTARY ARTICLES

Synthetic biology: insights into biological computation

Romilde Manzoni*, Arturo Urrios*, Silvia Velazquez-Garcia, Eulàlia de Nadal and Francesc Posas.

Integrative Biology 8.4 (2016): 518-532

* These authors have contributed equally to this work

REVIEW ARTICLE

Manzoni R, Urrios A, Velazquez-Garcia S, de Nadal E, Posas F. Synthetic biology: insights into biological computation. Integr Biol (Camb). 2016 Apr 18;8(4):518–32. DOI: 10.1039/c5ib00274e

The upcoming 3D-printing revolution in microfluidics

Nirveek Bhattacharjee, Arturo Urrios, Shawn Kang and Albert Folch

Lab on a Chip 16.10 (2016): 1720-1742

REVIEW ARTICLE

Bhattacharjee N, Urrios A, Kang S, Folch A. The upcoming 3D-printing revolution in microfluidics. Lab Chip. 2016;16(10):1720–42. DOI: 10.1039/C6LC00163G

3D-printing of transparent bio-microfluidic devices in PEG-DA

Arturo Urrios*, Cesar Parra-Cabrera*, Nirveek Bhattacharjee*, Alan M. Gonzalez-Suarez*, Luis G. Rigat-Brugarolas, Umashree Nallapatti, Josep Samitier, Cole A. DeForest, Francesc Posas, José L. Garcia-Cordero and Albert Folch.

Lab on a Chip 16.12 (2016): 2287-2294

* These authors have contributed equally to this work

RESULTS

Urrios A, Parra-Cabrera C, Bhattacharjee N, Gonzalez-Suarez AM, Rigat-Brugarolas LG, Nallapatti U, et al. 3D-printing of transparent bio-microfluidic devices in PEG-DA. Lab Chip. 2016;16(12):2287–94. DOI: 10.1039/C6LC00153J

3D-printing of transparent bio-microfluidic devices in PEG-DA

Arturo Urrios*, Cesar Parra-Cabrera*, Nirveek Bhattacharjee*, Alan M. Gonzalez-Suarez*, Luis G. Rigat-Brugarolas, Umashree Nallapatti, Josep Samitier, Cole A. DeForest, Francesc Posas, José L. Garcia-Cordero and Albert Folch.

Lab on a Chip 16.12 (2016): 2287-2294

* These authors have contributed equally to this work

DISCUSSION

In previous studies we demonstrated that multicellular synthetic circuits that combine distributed computation with spatial segregation performed complex computations with minimal cell engineering (Macia et al., 2016). From this study, we realized that by having custom microfluidic devices to isolate cells and control the extracellular environment our work could be further exploited to build more complex circuits. During my PhD thesis, I had the opportunity to do a stay abroad at the Folch lab (University of Washington, Seattle) which specializes in building automated custom microfluidic devices. When I arrived there they were setting up 3D-printing techniques to build microfluidic devices, in this context, I explored the use of 3D-printing to build transparent biocompatible fluidic devices (Urrios, Parra-Cabrera, et al., 2016) which can be further extended to build custom microfluidic devices.

Microfluidic systems are used in a broad range of biological applications (Balagaddé et al., 2005; Grilly et al., 2007; Groisman et al., 2005). Nowadays, they are built by replica-molding and bonding of elastomers (e.g. PDMS) or thermoplastics (e.g. polystyrene).

The widespread use of microfluidics devices is due to four main features: biocompatibility, optical transparency, low cost and the technology to produce micron-resolution features (Bhattacharjee et al., 2016). In addition, elastomers, such as PDMS can be used for fluidic automation (Thorsen et al., 2002; Unger et al., 2000) and it is gas permeable (Merkel et al., 2000), a key factor for cell culture applications because it allows gas exchange.

However, the problem is that building microfluidics devices require a lengthy manual procedure that involves manual layering and special equipment to assemble the final device making it not suitable for rapid prototyping. One interesting alternative is the use

of 3D printing technologies such as stereolithography (SL) to build microfluidic devices. SL allows for an assembly-free production. SL requires two main components: a photoactivable liquid resin that upon light gets polymerized and a 3D printer. The 3D printer has two key components: a focused laser beam or a digital light projector (DLP) that projects patterns of light onto the resin and causes its polymerization, and a moving platform (building plate) where the polymerized resin attaches, and it can move up or down after the layer is printed allowing for the following layer to be automatically printed.

In this study, we aim at defining a new method for 3D printing devices for cell culturing. We used a highly biocompatible material (PEGDA-250) and a 385nm DLP as a light source to build transparent biocompatible fluidic devices.

Choice of resin components

The resin was composed by two elements: a monomer that can be polymerized and a photoinitiator that is stimulated by light and causes the monomer to polymerize. As a monomer, we used PEGDA-250 a transparent biocompatible monomer, with high molecular weight PEGDAs such as PEGDA-500 or PEGDA-750 which behaved like hydrogels, the prints were water permeable and easy to crack. For the photoinitiator, we chose two non-expensive and biocompatible photoinitiators: Irgacure 819 and Irgacure 784. Irgacure 784 was discarded because it produced prints with a dark orange coloring while with Irgacure 819 were colorless at 0.4%.

The meaning of resolution in SL microfluidc fabrication

The resolution of SL microfluidic fabrication has two limitations. One is a technical limitation, for example Z resolution

depends on the precision of the stepper motors to move the building plate, while XY resolution depends on the DLPs pixel size. The second limitation depends on the capability of the resin to absorb light and cause polymerization without diffusion effects.

The challenge of printing with transparent resins

SL printers nowadays use visible light as light source (either 405nm lasers or DLP projectors) for security reasons and to reduce costs. However, we cannot use these systems to print transparent resins because visible light goes through transparent prints and a little amount is absorbed producing very poor Z resolution. This problem is extremely relevant in microfluidic production, for example, in case of building the roof of a channel, light can penetrate through the roof layer if not absorbed properly and it can crosslink the whole channel. This effect is analyzed using the Beer-Lambert law, that relates absorbance, wavelength and penetration. We found that a 385nm light source optimized Z resolution in comparison with a visible light DLP because Irgacure 819 had a higher absorbance at that wavelength

Transparent prints

Using a resin composed by PEGDA-250 + Irgacure 819 0.4% resulted in colorless prints however they were not transparent. To solve this problem, we modified the building plate surface. Building plate surfaces are rough to allow the newly polymerized print to attach, however this makes the 3D printed object to have rough surfaces that diffract light making it translucent instead of transparent. To solve this, we changed the building plate by a glass smooth surface, however new prints did not attach to this surface. To overcome this limitation the glass building plate

was treated with acrylates groups making the surface specifically adhesive to new prints.

Cytocompatibility of the prints

To test the biocompatibility of the devices, CHO cells and primary mouse hippocampal neurons were grown in 3D printed dishes (PEGDA-250 + Irgacure 819 0.4%). Biocompatibility was comparable to tissue culture polystyrene (TCPS). Additionally, this material allowed obtaining phase contrast and epifluorescence images with a comparable quality as those taken on TCPS. However, we found that the devices that have just finished the printing process can cause cytotoxicity, this was solved by exposing the prints to a UV bath of 12h ensuring that the whole device was fully cured, the observed cell death was caused by leakiness of uncured monomer.

These results show that the use of PEGDA-250 + Irgacure 819 together with techniques to reduce surface roughness allow to build transparent biocompatible fluidic devices. However, with this method, the resolution for printing a channel was limited to $1mm^2$ of cross section, in order to improve resolution, further studies need to be done.

Perspective

We have demonstrated that PEGDA-250 and Irgacure 819 can be 3D printed to generate transparent, biocompatible, microfluidic devices. However, there are two big caveats that need to be solved before 3D printing becomes the preferred choice to build microfluidic devices.

First, the resolution obtained using this method allowed us to print channels of $1mm^2$ of cross section and some features in the

order of 200 micron. These features are huge in comparison with the 1-2 micron resolution that can be achieved with PDMS molding and bonding techniques. This poor resolution limits the applicability of the devices. Resolution can be improved by new technological advancements such as more precise stepper motors and higher resolution DLPs that work with UV light; or by using other photoinitiators or resin compositions. We observed that the photoinitiators used in this study have higher absorbance at lower wavelengths (UV spectrum), however UV light damages the current DLPs microchips.

Second, the resin composition confers the final properties of the device. PEGDA-250 is rigid, biocompatible, transparent and gas impermeable. Since the material is not gas permeable, culturing cells in a 3D printed channel can require of additional methods to promote gas exchange otherwise cells may die or bubbles can block the channels. On the other hand, since the device is rigid it is difficult to make valves and do microfluidic automation, this can be solved by using monomers with elastomeric properties.

Taken together, this method solves the problems of transparency and biocompatibility of 3D printed devices but further studies need to be performed to solve the problems of resolution and gas permeability. Despite being a field in its infancy, 3D printing holds the promise of revolutionizing the field of microfluidics since it allows for rapid prototyping, it does not require manual assembly or special equipment (besides a 3D printer), the device can be fully designed with AutoCAD and can be computer modeled to test the dynamic properties prior building it, and moreover since the design is contained in a CAD file it can be shared and printed easily in other facilities around the world.

Personal contribution

My personal contribution in this study consisted in generating transparent 3D printed devices. I worked on CAD design, photoresin composition, surface treatment, 3D printing setup, methods for increasing Z-resolution and improving biocompatibility, as well as writing the manuscript.

REFERENCES

Ajo-Franklin, C. M., Drubin, D. a, Eskin, J. A., Gee, E. P., Landgraf, D., Phillips, I., & Silver, P. A. (2007). Rational design of memory in eukaryotic cells. *Genes & Development*, *21*(617), 2271–6. https://doi.org/10.1101/gad.1586107

Aldor, I. S., & Keasling, J. D. (2003). Process design for microbial plastic factories: Metabolic engineering of polyhydroxyalkanoates. *Current Opinion in Biotechnology*, *14*(5), 475–483. https://doi.org/10.1016/j.copbio.2003.09.002

Ariño, J., Ramos, J., & Sychrová, H. (2010). Alkali Metal Cation Transport and Homeostasis in Yeasts. *Microbiology and Molecular Biology Reviews : MMBR*, *74*(1), 95–120. https://doi.org/10.1128/MMBR.00042-09

Au, A. K., Bhattacharjee, N., Horowitz, L. F., Chang, T. C., & Folch, A. (2015). 3D-Printed Microfluidic Automation. *Lab on a Chip*, *15*(8), 1934–1941. https://doi.org/10.1039/c5lc00126a

Bacchus, W., Aubel, D., & Fussenegger, M. (2013). Biomedically relevant circuit-design strategies in mammalian synthetic biology. *Molecular Systems Biology*, *9*(1).

Balagaddé, F. K., You, L., Hansen, C. L., Arnold, F. H., & Quake, S. R. (2005). Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Science (New York, N.Y.)*, *309*(5731), 137–140. https://doi.org/10.1126/science.1109173

Bardwell, L. (2005). A walk-through of the yeast mating pheromone response pathway. *Peptides*, *26*(2), 339–350.

Bardwell, L., Cook, J. G., Chang, E. C., Cairns, B. R., & Thorner, J. (1996). Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Molecular and Cellular Biology*, *16*(7), 3637–3650.

Bhattacharjee, N., Urrios, A., Kang, S., & Folch, A. (2016). The upcoming 3D-printing revolution in microfluidics. *Lab on a Chip*, *16*(10), 1720–1742. https://doi.org/10.1039/C6LC00163G

Bisson, L. F., Coons, D. M., Kruckeberg, A. L., & Lewis, D. A. (1993). Yeast Sugar Transporters. *Critical Reviews in Biochemistry and Molecular Biology*, *28*(4), 259–308. https://doi.org/10.3109/10409239309078437

Blackwell, E., Kim, H.-J. N., & Stone, D. E. (2007). The pheromone-induced nuclear accumulation of the Fus3 MAPK in yeast depends on its phosphorylation state and on Dig1 and Dig2. *BMC Cell Biology*, *8*, 44. https://doi.org/10.1186/1471-2121-8-44

Boles, E., & Hollenberg, C. P. (1997). The molecular genetics of hexose transport in yeasts. *FEMS Microbiology Reviews*, *21*(1), 85–111.

Bonnet, J., Subsoontorn, P., & Endy, D. (2012). Rewritable digital data storage in live cells via engineered control of recombination directionality. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(23), 8884–9. https://doi.org/10.1073/pnas.1202344109

Bonnet, J., Yin, P., Ortiz, M. E., Subsoontorn, P., & Endy, D. (2013a). Amplifying Genetic Logic Gates. *Science*, *340*(6132), 599 LP-603.

Bonnet, J., Yin, P., Ortiz, M. E., Subsoontorn, P., & Endy, D. (2013b). Amplifying genetic logic gates. *Science (New York, N.Y.)*, *340*(6132), 599–603. https://doi.org/10.1126/science.1232758

Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., & Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science*, *259*(5102), 1760 LP-1763.

Brückner, S., Köhler, T., Braus, G. H., Heise, B., Bolte, M., & Mösch, H.-U. (2004). Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificity in yeast development. *Current Genetics*, *46*(6), 331–342. https://doi.org/10.1007/s00294-004-0545-1

Chang, L., & Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature*, *410*(6824), 37–40.

Chen, Q., & Konopka, J. B. (1996). Regulation of the G-protein-coupled alpha-factor pheromone receptor by phosphorylation. *Molecular and Cellular Biology*, *16*(1), 247–257.

Chen, R. E., & Thorner, J. (2007). Function and regulation in MAPK signaling pathways: Lessons learned from the yeast Saccharomyces cerevisiae. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1773*(8), 1311–1340. https://doi.org/https://doi.org/10.1016/j.bbamcr.2007.05.003

Chen, Y.-J., Liu, P., Nielsen, A. A. K., Brophy, J. A. N., Clancy, K., Peterson, T., & Voigt, C. A. (2013). Characterization of 582 natural and synthetic terminators and quantification of their design constraints. *Nat Meth*, *10*(7), 659–664.

Chen, Y. Y., Jensen, M. C., & Smolke, C. D. (2010). Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. *Proceedings of the National Academy of Sciences* , *107*(19), 8531–8536. https://doi.org/10.1073/pnas.1001721107

Chickarmane, V., Troein, C., Nuber, U. A., Sauro, H. M., & Peterson, C. (2006). Transcriptional dynamics of the

embryonic stem cell switch. *PLoS Computational Biology*, *2*(9), e123. https://doi.org/10.1371/journal.pcbi.0020123

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., … Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science (New York, N.Y.)*, *339*(6121), 819–23.

Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumours. *Biochemical Journal*, *23*(3), 536 LP-545.

Daber, R., Stayrook, S., Rosenberg, A., & Lewis, M. (2007). Structural Analysis of Lac Repressor Bound to Allosteric Effectors. *Journal of Molecular Biology*, *370*(4), 609–619. https://doi.org/10.1016/j.jmb.2007.04.028

Daniel, R., Rubens, J. R., Sarpeshkar, R., & Lu, T. K. (2013). Synthetic analog computation in living cells. *Nature*, *497*(7451), 619–23. https://doi.org/10.1038/nature12148

Dann, S. G., & Thomas, G. (2006). The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Letters*, *580*(12), 2821–2829. https://doi.org/10.1016/j.febslet.2006.04.068

de Nadal, E., Alepuz, P. M., & Posas, F. (2002). Dealing with osmostress through MAP kinase activation. *EMBO Reports*, *3*(8), 735–740. https://doi.org/10.1093/embo-reports/kvf158

DeVit, M. J., & Johnston, M. (1999). The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae. *Current Biology*, *9*(21), 1231–1241. https://doi.org/https://doi.org/10.1016/S0960-9822(99)80503-X

Din, M. O., Danino, T., Prindle, A., Skalak, M., Selimkhanov, J., Allen, K., … Hasty, J. (2016). Synchronized cycles of bacterial lysis for in vivo delivery. *Nature*, *536*(7614), 81–85.

Dohlman, H. G., & Slessareva, J. E. (2006). Pheromone Signaling Pathways in Yeast. *Science's STKE*, *2006*(364), cm6 LP-cm6.

Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E., & Thorner, J. (1996). Sst2, a negative regulator of pheromone signaling in the yeast Saccharomyces cerevisiae: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit). *Molecular and Cellular Biology*, *16*(9), 5194–5209.

Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K., & Matsumoto, K. (1994). MSG5, a novel protein phosphatase promotes adaptation to pheromone response in S. cerevisiae. *Embo J*, *13*.

Dowell, S. J., Bishop, A. L., Dyos, S. L., Brown, A. J., & Whiteway,

M. S. (1998). Mapping of a yeast G protein betagamma signaling interaction. *Genetics*, *150*(4), 1407–1417.

Elowitz, M. B., & Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature*, *403*(6767), 335–338.

Elowitz, M. B., & Leibler, S. (2000). A synthetic oscillatory network of transcriptional regulators. *Nature*, *403*(6767), 335–338. https://doi.org/10.1038/35002125

Endy, D. (2005). Foundations for engineering biology. *Nature*, *438*(7067), 449–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16306983

Farzadfard, F., & Lu, T. K. (2014). Genomically encoded analog memory with precise in vivo DNA writing in living cell populations. *Science*, *346*(6211), 1256–272. https://doi.org/10.1126/science.1256272

Farzadfard, F., & Lu, T. K. (2014). Genomically Encoded Analog Memory with Precise In vivo DNA Writing in Living Cell Populations. *Science (New York, N.Y.)*, *346*(6211), 1256272. https://doi.org/10.1126/science.1256272

Feldmann, H. (2012a). Introduction. In *Yeast* (pp. 1–3). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527659180.ch1

Feldmann, H. (2012b). Molecular Signaling Cascades and Gene Regulation. In *Yeast* (pp. 273–299). Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527659180.ch10

Ferrell, J. E., & Machleder, E. M. (1998). The Biochemical Basis of an All-or-None Cell Fate Switch in Xenopus Oocytes. *Science*, *280*(5365), 895 LP-898.

Ferrer-Miralles, N., Domingo-Espín, J., Corchero, J. L., Vázquez, E., & Villaverde, A. (2009). Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories*, *8*(1), 17. https://doi.org/10.1186/1475-2859-8-17

Furukawa, K., & Hohmann, S. (2013). Synthetic biology: lessons from engineering yeast MAPK signalling pathways. *Molecular Microbiology*, *88*(1), 5–19. https://doi.org/10.1111/mmi.12174

Furukawa, K., & Hohmann, S. (2015). A fungicide-responsive kinase as a tool for synthetic cell fate regulation. *Nucleic Acids Research*, *43*(14), 7162–70. https://doi.org/10.1093/nar/gkv678

Gagiano, M., Bauer, F. F., & Pretorius, I. S. (2002). The sensing of nutritional status and the relationship to filamentous growth in Saccharomyces cerevisiae. *FEMS Yeast Research*, *2*(4), 433–470. https://doi.org/10.1111/j.1567-1364.2002.tb00114.x

Gardner, T. S., Cantor, C. R., & Collins, J. J. (2000). Construction of

a genetic toggle switch in Escherichia coli. *Nature*, *403*(6767), 339–342.

Garrison, T. R., Zhang, Y., Pausch, M., Apanovitch, D., Aebersold, R., & Dohlman, H. G. (1999). Feedback Phosphorylation of an RGS Protein by MAP Kinase in Yeast. *Journal of Biological Chemistry* , *274*(51), 36387–36391. https://doi.org/10.1074/jbc.274.51.36387

Gossen, M., & Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(12), 5547–5551. https://doi.org/10.1073/pnas.89.12.5547

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., & Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science*, *268*(5218), 1766 LP-1769.

Gottesman, S., Roche, E., Zhou, Y., & Sauer, R. T. (1998). The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes & Development*, *12*(9), 1338–1347.

Grilly, C., Stricker, J., Pang, W. L., Bennett, M. R., & Hasty, J. (2007). A synthetic gene network for tuning protein degradation in Saccharomyces cerevisiae. *Molecular Systems Biology*, *3*(127), 127. https://doi.org/10.1038/msb4100168

Groisman, A., Lobo, C., Cho, H., Campbell, J. K., Dufour, Y. S., Stevens, A. M., & Levchenko, A. (2005). A microfluidic chemostat for experiments with bacterial and yeast cells. *Nature Methods*, *2*(9), 685–689. https://doi.org/10.1038/nmeth784

Hagen, D. C., McCaffrey, G., & Sprague, G. F. (1986). Evidence the yeast STE3 gene encodes a receptor for the peptide pheromone a factor: gene sequence and implications for the structure of the presumed receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(5), 1418–1422.

Hardie, D., Carling, D., & Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annual Review of Biochemistry*, *67*(1), 821–855.

Hay, C. W., & Docherty, K. (2003). Enhanced expression of a furin-cleavable proinsulin. *J Mol Endocrinol*, *31*(3), 597–607.

Hedbacker, K., & Carlson, M. (2008). SNF1/AMPK pathways in yeast. *Frontiers in Bioscience : A Journal and Virtual Library*, *13*, 2408–2420.

Hedges, D., Proft, M., & Entian, K. D. (1995). CAT8, a new zinc

cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *15*(4), 1915–1922.

Hirayarna, T., Maeda, T., Saito, H., & Shinozaki, K. (1995). Cloning and characterization of seven cDNAs for hyperosmolarity-responsive (HOR) genes of Saccharomyces cerevisiae. *Molecular and General Genetics MGG*, *249*(2), 127–138. https://doi.org/10.1007/BF00290358

Hohmann, S. (2002). Osmotic Stress Signaling and Osmoadaptation in Yeasts. *Microbiology and Molecular Biology Reviews*, *66*(2), 300–372. https://doi.org/10.1128/MMBR.66.2.300-372.2002

Hong, S.-P., Leiper, F. C., Woods, A., Carling, D., & Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(15), 8839–8843. https://doi.org/10.1073/pnas.1533136100

Inniss, M. C., & Silver, P. A. (2013). Building synthetic memory. *Current Biology*, *23*(17), R812-6. https://doi.org/10.1016/j.cub.2013.06.047

Isaacs, F. J., Hasty, J., Cantor, C. R., & Collins, J. J. (2003). Prediction and measurement of an autoregulatory genetic module. *Proc. Natl. Acad. Sci. U. S. A.*, *100*(13), 7714–7719.

Jacob, F., & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology*, *3*(3), 318–356. https://doi.org/http://dx.doi.org/10.1016/S0022-2836(61)80072-7

Jacob, F., & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol*, *3*, 318–56.

Jenkins, G. M. (2003). The emerging role for sphingolipids in the eukaryotic heat shock response. *Cellular and Molecular Life Sciences : CMLS*, *60*(4), 701–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12785717

Jiang, R., & Carlson, M. (1996). Glucose regulates protein interactions within the yeast SNF1 protein kinase complex. *Genes & Development* , *10*(24), 3105–3115. https://doi.org/10.1101/gad.10.24.3105

Johnston, M. (1999). Feasting, fasting and fermenting: glucose sensing in yeast and other cells. *Trends in Genetics*, *15*(1), 29–33. https://doi.org/https://doi.org/10.1016/S0168-9525(98)01637-0

Johnston, M., & Kim, J.-H. (2005). Glucose as a hormone: receptor-mediated glucose sensing in the yeast

Saccharomyces cerevisiae *Biochemical Society Transactions*, *33*(1), 247 LP-252.

Karzai, A. W., Roche, E. D., & Sauer, R. T. (2000). The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat Struct Mol Biol*, *7*(6), 449–455.

Kayikci, Ö., & Nielsen, J. (2015). Glucose repression in Saccharomyces cerevisiae. *FEMS Yeast Research*, *15*(6), fov068. https://doi.org/10.1093/femsyr/fov068

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M., & Johnson, A. D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell*, *68*(4), 709–719. https://doi.org/http://dx.doi.org/10.1016/0092-8674(92)90146-4

Khakhar, A., Bolten, N. J., Nemhauser, J., & Klavins, E. (2016). Cell–Cell Communication in Yeast Using Auxin Biosynthesis and Auxin Responsive CRISPR Transcription Factors. *ACS Synthetic Biology*, *5*(4), 279–286. https://doi.org/10.1021/acssynbio.5b00064

Kiani, S., Beal, J., Ebrahimkhani, M. R., Huh, J., Hall, R. N., Xie, Z., … Weiss, R. (2014). CRISPR transcriptional repression devices and layered circuits in mammalian cells. *Nature Methods*, (May), 1–5. https://doi.org/10.1038/nmeth.2969

Konopka, J. B., Jenness, D. D., & Hartwell, L. H. (1988). The C-terminus of the S. cerevisiae α-pheromone receptor mediates an adaptive response to pheromone. *Cell*, *54*(5), 609–620. https://doi.org/https://doi.org/10.1016/S0092-8674(88)80005-9

Kyriakis, J. M., & Avruch, J. (2001). Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation. *Physiological Reviews*, *81*(2), 807 LP-869.

Lafuente, M. J., Gancedo, C., Jauniaux, J.-C., & Gancedo, J. M. (2000). Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in Saccharomyces cerevisiae‡. *Molecular Microbiology*, *35*(1), 161–172. https://doi.org/10.1046/j.1365-2958.2000.01688.x

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews. Genetics*, *3*(9), 662–673. https://doi.org/10.1038/nrg887

Li, F. N., & Johnston, M. (1997). Grr1 of Saccharomyces cerevisiae is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and the cell cycle. *The EMBO Journal*, *16*(18), 5629–5638. https://doi.org/10.1093/emboj/16.18.5629

Li, Y., Jiang, Y., Chen, H., Liao, W., Li, Z., Weiss, R., & Xie, Z. (2015). Modular construction of mammalian gene circuits

using TALE transcriptional repressors. *Nature Chemical Biology*, *11*(3), 207–13. https://doi.org/10.1038/nchembio.1736

Liang, H., & Gaber, R. F. (1996). A novel signal transduction pathway in Saccharomyces cerevisiae defined by Snf3-regulated expression of HXT6. *Molecular Biology of the Cell* , *7*(12), 1953–1966. https://doi.org/10.1091/mbc.7.12.1953

Lin, B., & Levchenko, A. (2012). Microfluidic technologies for studying synthetic circuits. *Current Opinion in Chemical Biology*, *16*(3–4), 307–317. https://doi.org/10.1016/j.cbpa.2012.04.012

Lohr, D., Venkov, P., & Zlatanova, J. (1995). Transcriptional regulation in the yeast GAL gene family: a complex genetic network. *The FASEB Journal* , *9*(9), 777–787.

Lucks, J. B., Qi, L., Mutalik, V. K., Wang, D., & Arkin, A. P. (2011). Versatile RNA-sensing transcriptional regulators for engineering genetic networks. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(21), 8617–22. https://doi.org/10.1073/pnas.1015741108

Ma, D., Cook, J. G., & Thorner, J. (1995). Phosphorylation and localization of Kss1, a MAP kinase of the Saccharomyces cerevisiae pheromone response pathway. *Molecular Biology of the Cell*, *6*(7), 889–909.

Macia, J., Manzoni, R., Conde, N., Urrios, A., de Nadal, E., Solé, R., & Posas, F. (2016). Implementation of Complex Biological Logic Circuits Using Spatially Distributed Multicellular Consortia. *PLoS Computational Biology*, *12*(2), e1004685. https://doi.org/10.1371/journal.pcbi.1004685

Macía, J., Posas, F., & Solé, R. V. (2012). Distributed computation: the new wave of synthetic biology devices. *Trends in Biotechnology*, *30*(6), 342–9. https://doi.org/10.1016/j.tibtech.2012.03.006

Macía, J., Widder, S., & Solé, R. (2009). Why are cellular switches Boolean? General conditions for multistable genetic circuits. *Journal of Theoretical Biology*, *261*(1), 126–135. https://doi.org/10.1016/j.jtbi.2009.07.019

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., Dicarlo, J. E., … Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823–6.

Manney, T. R. (1983). Expression of the BAR1 gene in Saccharomyces cerevisiae: induction by the alpha mating pheromone of an activity associated with a secreted protein. *Journal of Bacteriology*, *155*(1), 291–301.

Manzoni, R., Urrios, A., Velazquez-Garcia, S., de Nadal, E., & Posas, F. (2016). Synthetic biology: insights into biological

computation. *Integrative Biology*, *8*(4), 518–532. https://doi.org/10.1039/C5IB00274E

Marshall-Carlson, L., Celenza, J. L., Laurent, B. C., & Carlson, M. (1990). Mutational analysis of the SNF3 glucose transporter of Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *10*(3), 1105–1115.

McKinney, J. D., & Cross, F. R. (1995). FAR1 and the G1 phase specificity of cell cycle arrest by mating factor in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *15*(5), 2509–2516.

Merkel, T. C., Bondar, V. I., Nagai, K., Freeman, B. D., & Pinnau, I. (2000). Gas sorption, diffusion, and permeation in poly(dimethylsiloxane). *Journal of Polymer Science Part B: Polymer Physics*, *38*(3), 415–434. https://doi.org/10.1002/(SICI)1099-0488(20000201)38:3<415::AID-POLB8>3.0.CO;2-Z

Merlini, L., Dudin, O., & Martin, S. G. (2013). Mate and fuse: how yeast cells do it. *Open Biology*, *3*(3), 130008. https://doi.org/10.1098/rsob.130008

Michaelis, S., & Barrowman, J. (2012). Biogenesis of the Saccharomyces cerevisiae pheromone a-factor, from yeast mating to human disease. *Microbiology and Molecular Biology Reviews : MMBR*, *76*(3), 626–51. https://doi.org/10.1128/MMBR.00010-12

Miller, C. A., Tan, X., Wilson, M., Bhattacharyya, S., & Ludwig, S. (2010). Single Plasmids Expressing Human Steroid Hormone Receptors and a Reporter Gene for Use in Yeast Signaling Assays. *Plasmid*, *63*(2), 73. https://doi.org/10.1016/j.plasmid.2009.11.003

Moon, T. S., Lou, C., Tamsir, A., Stanton, B. C., & Voigt, C. A. (2012). Genetic programs constructed from layered logic gates in single cells. *Nature*, *491*(7423), 249–253. https://doi.org/10.1038/nature11516

Moriya, H., & Johnston, M. (2004). Glucose sensing and signaling in Saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(6), 1572–1577. https://doi.org/10.1073/pnas.0305901101

Mosley, A. L., Lakshmanan, J., Aryal, B. K., & Özcan, S. (2003). Glucose-mediated Phosphorylation Converts the Transcription Factor Rgt1 from a Repressor to an Activator. *Journal of Biological Chemistry* , *278*(12), 10322–10327. https://doi.org/10.1074/jbc.M212802200

Naider, F., & Becker, J. M. (2004). The α-factor mating pheromone

of Saccharomyces cerevisiae: a model for studying the interaction of peptide hormones and G protein-coupled receptors. *Peptides*, *25*(9), 1441–1463. https://doi.org/https://doi.org/10.1016/j.peptides.2003.11.028

Nath, N., McCartney, R. R., & Schmidt, M. C. (2003). Yeast Pak1 Kinase Associates with and Activates Snf1. *Molecular and Cellular Biology*, *23*(11), 3909–3917. https://doi.org/10.1128/MCB.23.11.3909-3917.2003

Nehlin, J. O., Carlberg, M., & Ronne, H. (1991). Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. *The EMBO Journal*, *10*(11), 3373–3377.

Nevoigt, E. (2008). Progress in metabolic engineering of Saccharomyces cerevisiae. *Microbiology and Molecular Biology Reviews : MMBR*, *72*(3), 379–412. https://doi.org/10.1128/MMBR.00025-07

Nissim, L., Perli, S. D., Fridkin, A., Perez-Pinera, P., & Lu, T. K. (2014). Multiplexed and Programmable Regulation of Gene Networks with an Integrated RNA and CRISPR/Cas Toolkit in Human Cells. *Molecular Cell*, *54*(4), 698–710. https://doi.org/10.1016/j.molcel.2014.04.022

Nurse, P. (2008). Life, logic and information. *Nature*, *454*(7203), 424–426. https://doi.org/10.1038/454424a

Östling, J., & Ronne, H. (1998). Negative control of the Mig1p repressor by Snf1p-dependent phosphorylation in the absence of glucose. *European Journal of Biochemistry*, *252*(1), 162–168. https://doi.org/10.1046/j.1432-1327.1998.2520162.x

Ozcan, S., Dover, J., & Johnston, M. (1998). Glucose sensing and signaling by two glucose receptors in the yeast Saccharomyces cerevisiae. *The EMBO Journal*, *17*(9), 2566–2573. https://doi.org/10.1093/emboj/17.9.2566

Ozcan, S., & Johnston, M. (1995). Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. *Molecular and Cellular Biology*, *15*(3), 1564–72. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=230380&tool=pmcentrez&rendertype=abstract

Ozcan, S., & Johnston, M. (1996). Two different repressors collaborate to restrict expression of the yeast glucose transporter genes HXT2 and HXT4 to low levels of glucose. *Molecular and Cellular Biology* , *16*(10), 5536–5545. https://doi.org/10.1128/MCB.16.10.5536

Özcan, S., & Johnston, M. (1999). Function and Regulation of Yeast Hexose Transporters Function and Regulation of Yeast

Hexose Transporters, *63*(3).

Ozcan, S., Leong, T., & Johnston, M. (1996). Rgt1p of Saccharomyces cerevisiae, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Molecular and Cellular Biology*, *16*(11), 6419–26. https://doi.org/10.1128/MCB.16.11.6419

Paddon, C. J., Westfall, P. J., Pitera, D. J., Benjamin, K., Fisher, K., McPhee, D., … Newman, J. D. (2013). High-level semi-synthetic production of the potent antimalarial artemisinin. *Nature*, *496*(7446), 528–32. https://doi.org/10.1038/nature12051

Pagliuca, F. W., Millman, J. R., Gürtler, M., Segel, M., Van Dervort, A., Ryu, J. H., … Melton, D. A. (2014). Generation of functional human pancreatic β cells in vitro. *Cell*, *159*(2), 428–439. https://doi.org/10.1016/j.cell.2014.09.040

Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., & Keasling, J. D. (2012a). Microbial engineering for the production of advanced biofuels. *Nature*, *488*(7411), 320–328. https://doi.org/10.1038/nature11478

Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., & Keasling, J. D. (2012b). Microbial engineering for the production of advanced biofuels. *Nature*, *488*(7411), 320–328. Retrieved from http://dx.doi.org/10.1038/nature11478

Peyser, T., Dassau, E., Breton, M., & Skyler, J. S. (2014). The artificial pancreas: current status and future prospects in the management of diabetes. *Annals of the New York Academy of Sciences*, *1311*(1), 102–123. https://doi.org/10.1111/nyas.12431

Porro, D., Sauer, M., Branduardi, P., & Mattanovich, D. (2005). Recombinant protein production in yeasts. *Mol Biotechnol*, *31*. https://doi.org/10.1385/MB:31:3:245

Purcell, O., & Lu, T. K. (2014). Synthetic analog and digital circuits for cellular computation and memory. *Current Opinion in Biotechnology*, *29*, 146–55. https://doi.org/10.1016/j.copbio.2014.04.009

Qi, M., & Elion, E. A. (2005). MAP kinase pathways. *Journal of Cell Science*, *118*(16), 3569 LP-3572.

Qi, M., Kinzer, K., Danielson, K. K., Martellotto, J., Barbaro, B., Wang, Y., … Oberholzer, J. (2014). Five-year Follow-up of Patients with Type 1 Diabetes Transplanted with Allogeneic Islets: The UIC Experience. *Acta Diabetologica*, *51*(5), 833–843. https://doi.org/10.1007/s00592-014-0627-6

Ramezani-Rad, M. (2003). The role of adaptor protein Ste50-dependent regulation of the MAPKKK Ste11 in multiple

signalling pathways of yeast. *Current Genetics*, *43*(3), 161–70.
Regot, S., Macia, J., Conde, N., Furukawa, K., Kjellén, J., Peeters, T., … Solé, R. (2011). Distributed biological computation with multicellular engineered networks. *Nature*, *469*, 207–211. https://doi.org/10.1038/nature09679
Reifenberger, E., Boles, E., & Ciriacy, M. (1997). Kinetic Characterization of Individual Hexose Transporters of Saccharomyces Cerevisiae and their Relation to the Triggering Mechanisms of Glucose Repression. *European Journal of Biochemistry*, *245*(2), 324–333. https://doi.org/10.1111/j.1432-1033.1997.00324.x
Richardson, S. M., Mitchell, L. A., Stracquadanio, G., Yang, K., Dymond, J. S., DiCarlo, J. E., … Bader, J. S. (2017). Design of a synthetic yeast genome. *Science*, *355*(6329), 1040 LP-1044.
Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., … Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*, *440*(7086), 940–3. https://doi.org/10.1038/nature04640
Roquet, N., & Lu, T. K. (2014). Digital and analog gene circuits for biotechnology. *Biotechnology Journal*, *9*(5), 597–608. https://doi.org/10.1002/biot.201300258
Russell, S. J. (2015). Progress of artificial pancreas devices toward clinical use: the first outpatient studies. *Current Opinion in Endocrinology, Diabetes, and Obesity*, *22*(2), 106–111. https://doi.org/10.1097/MED.0000000000000142
Saito, H. (2010). Regulation of cross-talk in yeast MAPK signaling pathways. *Current Opinion in Microbiology*, *13*(6), 677–683. https://doi.org/https://doi.org/10.1016/j.mib.2010.09.001
Sanz, P., Alms, G. R., Haystead, T. A. J., & Carlson, M. (2000). Regulatory Interactions between the Reg1-Glc7 Protein Phosphatase and the Snf1 Protein Kinase. *Molecular and Cellular Biology*, *20*(4), 1321–1328.
Sardanyés, J., Bonforti, A., Conde, N., Solé, R., & Macia, J. (2015). Computational implementation of a tunable multicellular memory circuit for engineered eukaryotic consortia . *Frontiers in Physiology* . Retrieved from http://journal.frontiersin.org/article/10.3389/fphys.2015.00281
Sauro, H. M., & Kim, K. H. (2013). Synthetic biology: It's an analog world. *Nature*, *497*, 572–573. https://doi.org/10.1038/497573a
Segall, J. E. (1993). Polarization of yeast cells in spatial gradients of alpha mating factor. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(18), 8332–8336.

Sha, W., Moore, J., Chen, K., Lassaletta, A. D., Yi, C. S., Tyson, J. J., & Sible, J. C. (2003). Hysteresis drives cell-cycle transitions in Xenopus laevis egg extracts. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(3), 975–980. https://doi.org/10.1073/pnas.0235349100

Shao, J., Xue, S., Yu, G., Yu, Y., Yang, X., Bai, Y., … Ye, H. (2017). Smartphone-controlled optogenetically engineered cells enable semiautomatic glucose homeostasis in diabetic mice. *Science Translational Medicine*, *9*(387).

Shaw, J. E., Sicree, R. A., & Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice*, *87*(1), 4–14. https://doi.org/http://dx.doi.org/10.1016/j.diabres.2009.10.007

Siuti, P., Yazbek, J., & Lu, T. K. (2013). Synthetic circuits integrating logic and memory in living cells. *Nature Biotechnology*, *31*(5), 448–52. https://doi.org/10.1038/nbt.2510

Stricker, J., Cookson, S., Bennett, M. R., Mather, W. H., Tsimring, L. S., & Hasty, J. (2008). A fast, robust and tunable synthetic gene oscillator. *Nature*, *456*(7221), 516–519.

Tabor, J. J., Salis, H. M., Simpson, Z. B., Chevalier, A. A., Levskaya, A., Marcotte, E. M., … Ellington, A. D. (2009). A synthetic genetic edge detection program. *Cell*, *137*(7), 1272–81. https://doi.org/10.1016/j.cell.2009.04.048

Takahashi, S., & Pryciak, P. M. (2008). Membrane localization of scaffold proteins promotes graded signaling in the yeast MAP kinase cascade. *Current Biology : CB*, *18*(16), 1184–1191. https://doi.org/10.1016/j.cub.2008.07.050

Tamsir, A., Tabor, J. J., & Voigt, C. A. (2011a). Robust multicellular computing using genetically encoded NOR gates and chemical “wires.” *Nature*, *469*(7329), 212–215. https://doi.org/10.1038/nature09565

Tamsir, A., Tabor, J. J., & Voigt, C. A. (2011b). Robust multicellular computing using genetically encoded NOR gates and chemical “wires”. *Nature*, *469*(7329), 212–215. https://doi.org/10.1038/nature09565

Thorsen, T., Maerkl, S. J., & Quake, S. R. (2002). Microfluidic Large-Scale Integration. *Science*, *298*(5593), 580 LP-584. Retrieved from http://science.sciencemag.org/content/298/5593/580.abstract

Treitel, M. A., Kuchin, S., & Carlson, M. (1998). Snf1 Protein Kinase Regulates Phosphorylation of the Mig1 Repressor in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, *18*(11), 6273–6280.

Trumbly, R. J. (1992). Glucose repression in the yeast

Saccharomyces cerevisiae. *Molecular Microbiology*, *6*(1), 15–21. https://doi.org/10.1111/j.1365-2958.1992.tb00832.x

Tschopp, J. F., Emr, S. D., Field, C., & Schekman, R. (1986). GAL2 codes for a membrane-bound subunit of the galactose permease in Saccharomyces cerevisiae. *Journal of Bacteriology*, *166*(1), 313–318.

Tu, J., & Carlson, M. (1995). REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. *The EMBO Journal*, *14*(23), 5939–5946.

Unger, M. A., Chou, H.-P., Thorsen, T., Scherer, A., & Quake, S. R. (2000). Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography. *Science*, *288*(5463), 113 LP-116. Retrieved from http://science.sciencemag.org/content/288/5463/113.abstract

Urrios, A., Macia, J., Manzoni, R., Conde, N., Bonforti, A., de Nadal, E., … Solé, R. (2016). A Synthetic Multicellular Memory Device. *ACS Synthetic Biology*, *5*(8), 862–873. https://doi.org/10.1021/acssynbio.5b00252

Urrios, A., Parra-Cabrera, C., Bhattacharjee, N., Gonzalez-Suarez, A. M., Rigat-Brugarolas, L. G., Nallapatti, U., … Folch, A. (2016). 3D-Printing of transparent bio-microfluidic devices in PEG-DA. *Lab on a Chip*, *16*(12), 2287–2294. https://doi.org/10.1039/c6lc00153j

Vincent, O., & Carlson, M. (1998). Sip4, a Snf1 kinase-dependent transcriptional activator, binds to the carbon source-responsive element of gluconeogenic genes. *The EMBO Journal*, *17*(23), 7002–7008. https://doi.org/10.1093/emboj/17.23.7002

Wang, Y., Yau, Y. Y., Perkins-Balding, D., & Thomson, J. G. (2011). Recombinase technology: Applications and possibilities. *Plant Cell Reports*, *30*(3), 267–285. https://doi.org/10.1007/s00299-010-0938-1

Warburg, O., Wind, F., & Negelein, E. (1927). THE METABOLISM OF TUMORS IN THE BODY. *The Journal of General Physiology*, *8*(6), 519–530.

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C. P., & Boles, E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in Saccharomyces cerevisiae. *FEBS Letters*, *464*(3), 123–128. https://doi.org/10.1016/S0014-5793(99)01698-1

Win, M. N., & Smolke, C. D. (2008). Higher-Order Cellular Information Processing with Synthetic RNA Devices. *Science*, *322*(5900), 456–460. https://doi.org/10.1126/science.1160311.Higher-Order

Wu, C.-Y., Roybal, K. T., Puchner, E. M., Onuffer, J., & Lim, W. A. (2015). Remote control of therapeutic T cells through a small molecule–gated chimeric receptor. *Science*, *350*(6258).

Xie, M., Ye, H., Wang, H., Charpin-El Hamri, G., Lormeau, C., Saxena, P., … Fussenegger, M. (2016). β-cell–mimetic designer cells provide closed-loop glycemic control. *Science*, *354*(6317), 1296 LP-1301.

Xie, Z.-X., Li, B.-Z., Mitchell, L. A., Wu, Y., Qi, X., Jin, Z., … Yuan, Y.-J. (2017). “Perfect” designer chromosome V and behavior of a ring derivative. *Science*, *355*(6329).

Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., & Benenson, Y. (2011). Multi-Input RNAi-Based Logic Circuit for Identification of Specific Cancer Cells. *Science*, *333*(6047), 1307–11.

Ye, H., Charpin-El Hamri, G., Zwicky, K., Christen, M., Folcher, M., & Fussenegger, M. (2013). Pharmaceutically controlled designer circuit for the treatment of the metabolic syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(1), 141–6. https://doi.org/10.1073/pnas.1216801110

Ye, H., Daoud-El Baba, M., Peng, R. W., & Fussenegger, M. (2011). A synthetic optogenetic transcription device enhances blood-glucose homeostasis in mice. *Science (New York, N.Y.)*, *332*(6037), 1565–8. https://doi.org/10.1126/science.1203535

Yin, Z., Wilson, S., Hauser, N. C., Tournu, H., Hoheisel, J. D., & Brown, A. J. P. (2003). Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs. *Molecular Microbiology*, *48*(3), 713–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12694616

Zheng, W., Zhao, H., Mancera, E., Steinmetz, L. M., & Snyder, M. (2010). Genetic Analysis of Variation in Transcription Factor Binding in Yeast. *Nature*, *464*(7292), 1187–1191. https://doi.org/10.1038/nature08934

Zhou, Y. J., Buijs, N. A., Zhu, Z., Qin, J., Siewers, V., & Nielsen, J. (2016). Production of fatty acid-derived oleochemicals and biofuels by synthetic yeast cell factories. *Nature Communications*, *7*, 11709.