Development of synthetic biology devices for iron metabolism research

Marco Salbany Constante Pereira

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THESIS SUPERVISOR

Dr. Mark Isalan

(EMBL/CRG Research Unit in Systems Biology)

DEPARTAMENT DE CIÈNCIES EXPERIMENTALS I DE LA SALUT

UNIVERSITAT POMPEU FABRA

ABSTRACT

Synthetic biology is a fairly recent field that aims to engineer novel functions in biological systems. In a broad sense synthetic biology encompasses the development of tools that makes the engineering of biology easier. In this thesis I develop a collection of standard DNA parts (Biobricks) that consists of a tool to build custom eukaryotic plasmids. This is not just intended for biology researchers in the field of synthetic biology, but also for more general use. Besides the development of molecular biology tools that facilitate the engineering of biology, synthetic biology researchers have implemented devices that are electronics-like in behavior and have demonstrated the potential of the field for the production of biofuels, pharmaceutics and biosensors. Here I present a sensor of iron regulatory protein activity, based on Biobricks. To demonstrate its use I apply it to the study of a novel reconstituted two cell-type co-culture (BNL CL.2 and RAW 264.7), surrogate for hepatocyte-macrophage communication.

RESUMEN

La biología sintética es un campo recientemente desarrollado con el objectivo de implementar nuevas funciones en sistemas biológicos. De forma global, la biología sintética incluye el desarrollo de herramientas para facilitar la ingeniería de sistemas biológicos. En diversas publicaciones, investigadores en el campo de la biología sintética han implementado dispositivos que funcionan de forma similar a circuitos electrónicos y han demonstrado el potencial del campo para la producción de biocarburantes, farmaceuticos y biosensores. Para la presente tesis he creado una colección de plasmidos estandarizados (Biobricks) que pueden ser de interés para biólogos fuera del campo da la biología sintética. Además, utilizando estos Biobricks, he creado un sensor de la actividad de las proteínas reguladas por el hierro. Para demonstrar su aplicación, he utilizado el sensor para estudiar un nuevo sistema de co-cultura de dos tipos celulares (BNL CL.2 y RAW 264.7), substituto para la comunicación entre hepatocitos y macrófagos.

1. INTRODUCTION

I provide here a brief introduction to the fields of synthetic biology and iron metabolism, around which the present thesis centers. As each of the resulting papers contains its own specialised introduction, this section provides a more general contextualization for the reported papers.

1.1 Synthetic Biology

Synthetic Biology is a fairly recent field (see section **The first landmarks**) that aims to engineer novel functions in biological systems. It distinguishes itself from other related fields, such as biotechnology, through the focus on modeling, whereby predictive power is gained over the designed systems. However, in a broad sense, the development of tools that makes the engineering of biology easier is also a key part of synthetic biology. In spite of the proximity of synthetic biology to biotechnology, it is more often put alongside the field of systems biology, where researchers, through modeling, try to represent existing natural systems and predict the effect of perturbations to the system. However, the marked contrast with systems biology is the component of engineering in synthetic biology.

Therefore one would ideally think of a synthetic biology project with an engineering framework in mind (figure 1.1), whereby a set of biological modules are created and characterized (see section **Parts and Devices**), allowing the computational modeling of the system (see section **Modeling**). Knowing the required constraints through modeling, a prototype is built to achieve a final product (see section **Applications**), although iterations of adjustments to the prototype are often required.

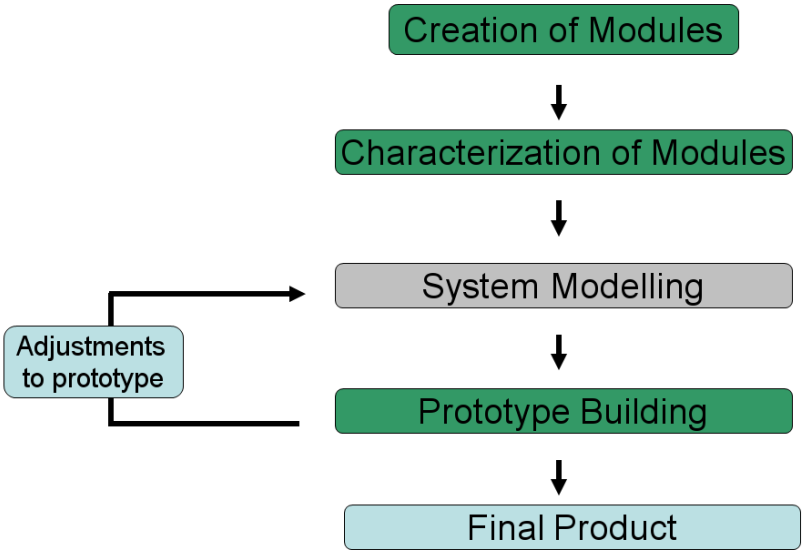

Figure 1.1 – **The engineering paradigm in synthetic biology.**

a) The first landmarks.

Two works from a decade ago are considered the landmarks of synthetic biology: a genetic toggle switch by Gardner *et al* (1) and a synthetic oscillator by Elowitz & Leibler (2).

Gardner *et al* (1) integrated theory and experiment by modeling the conditions for which a two-component toggle switch may exist and by constructing a couple of variations of such a switch. They used gene components which have appeared over-and-over again in subsequent synthetic biology studies (e.g., the tetracycline repressor (TetR) and the Lac I repressor (lacI) with IPTG as inducer 1 and anhydrotetracycline (aTC) as inducer 2; see figure 1.2A). For quantification they placed a green fluorescent protein (GFP) under the same promoter as TetR. As intended, the authors were able to add either of the inducers and toggle the switch into the corresponding stable state, which could be maintained even after the removal of the inducer.

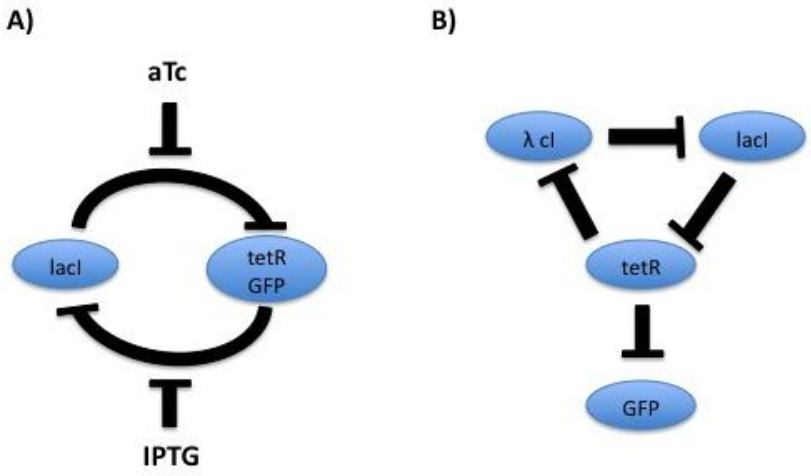

Figure 2. **The first landmark synthetic networks.** A) A toggle switch with aTc and IPTG as switching inducers. B) A three-leafed oscillatory network (repressilator).

In a slightly more complicated system, using three network nodes instead of two, Elowitz & Leibler (2) set out to engineer a system exhibiting oscillatory behavior. They made use of three components not found in natural oscillators: the tet repressor (TetR), the lac I repressor and the lambda phage cl repressor. By creating a network in a three leaf format (represented in figure 1.2B), where each repressor inhibits expression of the next leaf, and by using GFP under a promoter repressed by TetR as a reporter, they obtained oscillations of 160 ± 40 min, at the unicellular level.

In this thesis we model similar small networks and create ‘parts’ and ‘devices’ that help in engineering them.

b) Parts and Devices.

Since DNA is where the information is stored for the functioning of the cell, it is often through DNA that biological machines are ‘programmed’ (although exceptions exist, such as projects using lipid vesicles instead of live cells; see (3) for a review on engineering at different levels of complexity of components). When present, the cellular housing of a biological machine is referred to as the ‘chassis’ in synthetic biology terminology. The chassis is most often the

prokaryote *E. coli*, although Mycobacteriae, yeast or eukaryotic cell lines are also commonly used.

The engineering in these types of chassis is possible, in large part, thanks to our capacity to create and modify DNA with a set of techniques that have greatly evolved in recent years (see (4) for a recent review). The rapid decline in the cost of commercial synthesis (5) allows researchers to obtain easily a starting set of DNA molecules that may be further manipulated by fusing them. Fusion techniques include, for instance, *in vitro* thermocycled assembly based on the combined use of exonuclease III, Taq polymerase and Taq ligase (6). This enables the fusion of fragments from a few bp up to 318 kb, the approximate upper limit for transformation in *E. coli.* Alternatively, *in vivo* yeast transformation-assisted recombination has reported fusions of up to 583 kb using the the *M. genitalium* genome (6). In addition, an interesting new approach for DNA fusion came from the MIT in 2003 with a norm for standard biological 'parts' termed Biobricks.

Biobricks employ a pairwise 'idempotent' assembly method (assembly reactions are prepared two-by-two and leave the key elements unchanged). They may therefore be used iteratively to make a composition of any combination of Biobricks. The system is based on a specific prefix and suffix that contain restriction sites for a set of enzymes that, when ligated, produce a 'scar' sequence. The downsides of the pairwise system are that other fusion methods allow the fusion of many fragments at once and that the scar sequence can potentially interfere with the desired biological activity. However, these drawbacks are compensated by the fact that the process is always the same and can be semi-automated (a semi-automated streamlined protocol can be found in **Suplementary Article 1**). Moreover, no primers are required (in contrast to most of other fusion methods), reducing the cost of each assembly. Biobrick construction iterations easily allow for combinatorial arrangements (AB and BA are produced in the same manner without needing different sets of primers). Ultimately, the concept of the Biobrick standard brought not only a method for fusion of two segments of DNA, but it came with a strongly attached sense of 'Part'.

A Biobrick Part often has a particular function by itself, e.g. it may be a promoter or a ribosomal binding site (although a function is not a requirement). The online catalog of Biobrick Parts (http://partsregistry.org), or 'Registry', therefore allows not only for a researcher to find a

Biobrick that may be obtained, but also its sequence and, ideally, a functional characterization (e.g. Biobrick BBa_I0500, a characterized Inducible pBad/araC promoter).

Several of these parts may be assembled together to obtain a simple functional unit (a 'Device') such as the Biobrick BBa_I13263: a device composed of 10 individual parts designed to serve as a detection and quantification system for the concentration of the signal molecule N-Acyl homoserine lactone (AHL) in the growth medium.

However, it should be noted that the tools developed for synthetic biology may also be of use for researchers from other fields and in this thesis I present a Biobrick library of general use for cloning custom eukaryotic plasmids (**Article 1**). In the context of synthetic biology, the collection focuses on eukaryotic parts, which have been largely ignored in the Registry, and I go on to give several examples of functional synthetic contructs that can be built with this library.

The characterization of parts and devices is particularly important for what should really be the first step in the engineering of a larger biological network: the modeling of the system.

c) Modeling.

System modeling is an important part of synthetic biology and it is what makes it a truly interdisciplinary field (see (7) for a recent review on modeling and computational design). Often the modeling efforts for engineering in synthetic biology are performed between groups with complementary expertise or in groups that combine experimental 'wet labs' and theoretical 'dry labs'.

Biologists are used to representing pathways of interacting components with static arrow diagrams and, although this is filled with important information, it misses the dynamic properties of the system and normally does not provide the particular parameters of the network (the values that do not typically change over time such as binding constants). In addition, the particular architecture of a system (e.g. whether the repression of B by A is through an A monomer or by an AA dimer) is also usually absent in detail and this may give rise to different behaviors of the system, depending on the parameters (see **Supplementary Article 2** for an example).

Often, modeling of the desired system is difficult because the architecture and parameters of some of the modules used are not well-characterized. In many cases, this is simply due to the experimental difficulties of elucidating the values and mechanisms of the parts and devices used.

In the absence of formal characterization, educated guesses of the parameters and architectures usually have to be made and, ideally, a subsequent *in silico* analysis of the sensitivity of the system to changes in the guessed elements is performed. However, depending on the number of unknowns, the computational burden to perform such types of analysis may be prohibitive.

Software for computer assisted design (CAD), such as is often found in 'classical' engineering, is now being developed for synthetic biology such as the GenoCAD which allows a user to assemble Biobrick parts virtually into a device, using a graphical user interface (GUI) (8). Although still in its infancy, one may envision a future where, with the proper characterization of the component parts, a researcher may use a GUI-enabled CAD tool with integrated semi-automatic modeling to engineer the system for the desired application.

To encourage and develop synthetic biology engineering, an undergraduate competition has been started for international genetically engineered machines (iGEM) (see http://igem.org) (see (9) for a commentary on this competition). Since 2004, undergraduate student teams in iGEM use and contribute new Biobricks to the rapidly growing Registry, to build biological systems over a summer. The success of this endeavor is demonstrated not only by the growing number of teams participating, starting with 5 teams in 2004 and now reaching 165 teams in 2011, but also by the designed projects ranging from biological 'photographic' film (Texas team, 2004) to bacteria capable of transporting oxygen intended as a futuristic blood substitute (Berkeley team, 2007) or a DNA guided 'assembly line' whereby a DNA sequence is used as a scaffold for the binding of several zinc finger-fused enzymes (Slovenia team, 2010).

d) Applications.

Besides electronics-like programming (such as genetic switches (1,10-17), oscillators (2,10,18-21), digital logic gates (22-27), filters (28-30) and time-delayed circuits (31-33)), the field of synthetic biology brings large promises ranging from the production of biofuels, to pharmaceutical engineering of drugs or design of biosensors of diverse environmental signals

(See (34) and (35) for reviews on applications). For example, in this thesis, I present an Iron Regulatory Protein binding sensor, based on Biobricks, to study a novel reconstituted two cell-type (BNL CL.2 and RAW 264.7) co-culture (**Article 2**). As this system focuses on mamalian iron metabolism, I provide a general introduction to the topic in the following section.

1.2 Iron Metabolism

Although iron is required for a wide array of metabolic functions, it can be toxic to the cell through its participation in the formation of hydroxyl radicals that damage DNA, proteins and lipids. The duality of iron as an essential micronutrient and as a cellular toxin has imposed the evolution of highly-regulated systems for cellular iron uptake and storage in a non-toxic form, as discussed below in the section **Cellular iron**.

At the systemic level, the iron in a body is recycled from senescent cells and absorption is required to compensate for losses through desquamation of gastrointestinal cells, bleeding and other minor causes (36). This systemic iron recycling and regulation of absorption by the hormone hepcidin are discussed below in the section **Systemic iron**.

a) Cellular iron.

The main source of iron for mammalian cells is from transferrin (Tf) – synthesized mainly by hepatocytes (37) – by uptake through the Transferrin receptor (TfR) 1 or 2. To maintain cellular iron reserves in a non-toxic form, iron is loaded into the iron storage protein, ferritin. Non ferritin-bound intracellular iron constitutes the labile iron pool (LIP), which modulates the activity of iron regulatory proteins (IRPs).

Iron regulatory proteins (IRPs) 1 and 2 are messenger ribonucleic acid (mRNA) binding proteins with high affinities for hairpin secondary structures known as iron responsive elements (IREs) (38). IREs are present on the untranslated regions (UTRs) of several genes coding for proteins implicated in iron metabolism, including the TfR and ferritin polypeptides (38) and inhibit translation when present in the 5'UTR, whereas they protect RNA from degradation when present at the 3'UTR (see figure 1.3). I make use of this endogenous system to engineer a synthetic iron reporter system in **Article 2**.

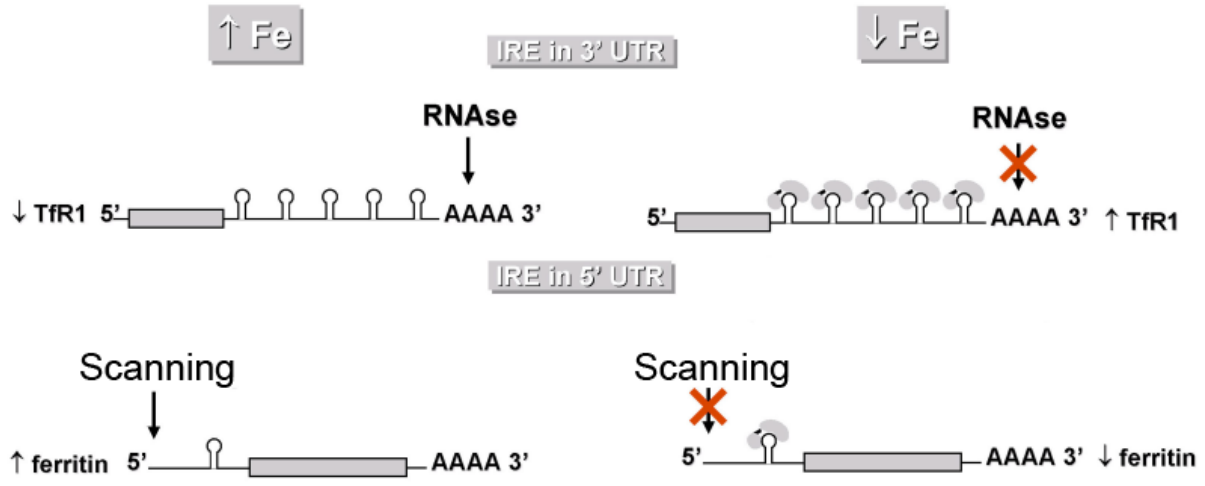

Figure 1.3 – **Responses to the labile iron pool mediated by IRE-IRP interactions.** Iron regulatory proteins (IRPs) bind to iron responsive elements (IREs) in low iron conditions and leave IREs free in the presence of iron. IRPs bound to IREs on the 3' UTR (as found in the Tf receptor 1 (TfR1) mRNA transcript), will block mRNA degradation by RNases. IRPs bound to 5' IREs (as found in ferritin) inhibit protein synthesis by disrupting ribosome scanning.

b) Systemic iron.

From the 3-5 g of iron present in the human body, approximately 1.8 g is found in hemoglobin in the erythrocytes, which have a life span of 120 days (39). To compensate for senescent erythrocytes, over 200 billion new erythrocytes are produced daily (40), making erythropoiesis the major iron-demanding process in the body.

To provide for erythropoiesis demand, iron is recycled. Macrophages phagocytose senescent RBCs and the free iron may then be exported into the bloodstream from the macrophages by ferroportin 1 (Fp1), to be bound to Tf (40). Subsequently the bone marrow uptakes the Tf-bound iron to fulfill its daily requirements.

Proportionally, only a very small amount of iron is taken up by the remaining cells for physiological use, of which muscle cells are the major players, containing iron in the form of myoglobin (36). The other exception is that of the liver. Hepatocytes have very high amounts of ferritin-associated iron and the liver is therefore regarded as an iron storage compartment, supplying iron in times of dietary iron deficiency (36).

In summary, cells may be regarded as iron 'acceptors' or 'donors'. Virtually all the cells in the body are iron 'acceptors', with erythropoietic precursor cells and hepatocytes as the main iron-demanding cells. The cell types that are considered to be iron 'donors' are 1) macrophages, after scavenging of iron from hemoglobin; 2) hepatocytes, in conditions of iron sparseness; and 3) enterocytes, responsible for absorbing iron in the duodenum (see figure 1.4 for a schematic representation).

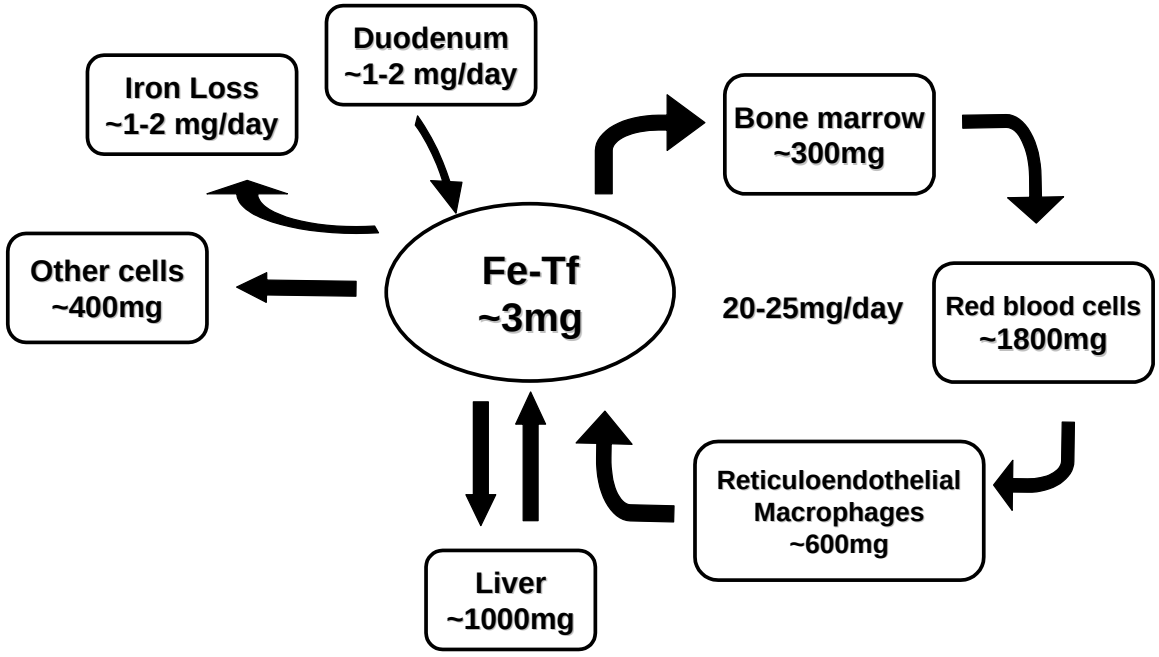

Figure 1.4 – **Systemic iron re-cycling.** Iron losses have to be compensated by iron absorption at the duodenum. Absorbed iron is released into the bloodstream where it is bound to Transferrin (Tf). Erythroid precursors uptake the Tf-iron to produce RBCs. Senescent RBCs are phagocytosed by the reticuloendothelial macrophages and the iron is recycled back into the bloodstream. Hepatocytes store excess iron and supply it back when systemic iron levels are reduced.

The recycling of iron implies that dietary iron requirements are only needed to compensate for iron losses and to support body growth. Given the nonexistence of a controlled iron excretion system, systemic iron levels must be regulated at the level of iron absorption.

Dietary iron is absorbed at the level of the duodenum and, after being loaded into the enterocytes, it is released into the bloodstream through the iron exporter Fp1, regulated by the hormone hepcidin, which is produced mainly in the liver. Hepcidin (see (41) for a review) was discovered a decade ago and it has since been shown to be regulated by iron levels,

erythropoietic demand, oxygen levels and inflammation (42) – the same conditions previously determined to be regulators of iron absorption (43).

Hepcidin is now regarded as the master regulator of iron metabolism and it has been shown that its effects are mediated by the inhibition of the iron exporter Fp1. Regulation of hepcidin will therefore eventually affect all iron 'donor' cells, expressing Fp1, including not only the enterocytes, but also the hepatocytes themselves and macrophages, thereby affecting iron homeostasis in general.

2. ARTICLE 1

A BIOBRICK LIBRARY FOR CLONING CUSTOM EUKARYOTIC PLASMIDS

Constante M, Grünberg R, Isalan M. A Biobrick Library for Cloning Custom Eukaryotic Plasmids. Plos One. 2011 6(8): e23685. doi:10.1371/journal.pone.0023685

3. ARTICLE 2

BNL CL.2 CELLS INCREASE IRP BINDING WHEN CO-CULTURED WITH RAW 264.7 MACROPHAGES: A SURROGATE MODEL FOR HEPATOCYTE-KUPFFER CELL COMMUNICATION

Constante M, Munteanu A, Santos MM, Isalan M.

UBNL CL.2 cells increase IRP binding when co-cultured with RAW 264.7 macrophages: a surrogate model for hepatocyte-Kupffer cell communication

Marco Constante[1§], Andreea Munteanu[1], Manuela M. Santos[2] & Mark Isalan[1]

[1]EMBL/CRG Systems Biology Research Unit, Centre for Genomic Regulation (CRG) and UPF Barcelona, Spain. [2]Department of Medicine, Faculty of Medicine, Université de Montréal, and Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM), Montréal, Québec, Canada

[§]To whom correspondence should be addressed

marco.constante@crg.es

andreea.munteanu@crg.es

manuela.santos@umontreal.ca

mark.isalan@crg.es

Abstract

In the present work we establish a transiently transfectable dual-fluorescence reporter, based on iron regulatory protein (IRP) binding activity, that inversely correlates with available iron levels in the medium. We verify this reporter by demonstrating changes in IRP binding activity under various conditions. For example, *E. coli* lipopolysaccharide (LPS) causes macrophages to release cytokines that increase iron levels in hepatocytes and we show that this is also true for BNL CL.2 liver cells treated with medium from RAW 264.7 cells, exposed to LPS. We further show that BNL CL.2 cells exhibit an increase in IRP binding activity when co-cultured with RAW 264.7 macrophages, in a surrogate system that stands in for hepatocyte-Kupffer cell co-culture. We investigate this regulation by assessing the gene expression profile using microarray analysis of BNL CL.2 cells alone or when co-cultured with RAW 264.7 (with and without addition of iron in the medium). This system provides a platform for addressing how macrophages participate in the iron homeostasis of liver cells and, ultimately, in systemic iron metabolism.

Introduction

Iron is an essential micronutrient for several essential biological processes, ranging from the production of ATP in the mitochondria to oxygen transport in the blood (44,45). This versatility is due to its capacity to form a variety of coordination complexes with organic ligands which, along with its ability to switch between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states, has allowed for the evolution of several functionally-diverse iron proteins (45). However, this propensity of iron to participate in oxidation-reduction reactions also accounts for the spontaneous reaction where iron catalyses the formation of hydroxyl radicals; these

react readily with DNA, proteins and lipids, inducing mutations and cellular stress (46). The cytoplasmic iron that is able to form toxic hydroxyl radicals is present in a form that is weakly chelated by a variety of organic ligands and is generally referred to as the labile iron pool (LIP) (47,48). The duality of iron as an essential micronutrient and as a cellular toxin has therefore imposed the evolution of highly-regulated systems for the balance of LIP levels.

Iron balance is modulated at the cellular level by the activity of iron regulatory proteins (IRPs)(49). These are mRNA-binding proteins with high affinity for hairpin secondary structures known as iron responsive elements (IREs). Whereas IREs in the 5'UTR (untranslated region) inhibit translation when IRPs are bound, IREs in the 3'UTR augment mRNA levels and translation by blocking degradation, when bound by IRPs (50). Two distinct IRPs, IRP1 and IRP2, bind IREs. Although structurally similar, the IRPs are regulated very differently: IRP1 contains an iron-sulfur 4Fe-4S cluster that loses one iron ion in low LIP levels, inducing IRE binding (51). Conversely, IRP2 is regulated by iron-induced degradation (52). In summary, IRPs will predominantly bind IREs when LIP levels are low, i.e., IRP binding typically inversely correlate with iron levels, although other regulatory mechanisms exist, such as the regulation of IRP binding activity by nitric oxide (53).

In addition to the iron balance achieved through modulation of IRP activity at the cellular level, a systemic iron regulation is also required. Since there is no specific mechanism for iron excretion, systemic iron levels have to be regulated through the control of iron absorption. Hepcidin, a small peptide hormone expressed mainly in the liver, has been found to inhibit the iron exporter ferroportin 1 (Fp1), by inducing its internalization and degradation (54) and reducing mRNA levels (43). This inhibits the release of iron from the duodenal enterocytes, responsible for iron absorption. The importance of hepcidin in the regulation of systemic iron levels has been confirmed using mice deficient for hepcidin (which are iron overloaded (55)) and transgenic mice overexpressing hepcidin (which are severely iron deficient (56)).

Besides its role in the regulation of iron absorption, hepcidin is also implicated in iron homeostasis in other systems and it participates in the inhibition of Fp1 at the level of macrophages, responsible for the release of iron recycled from senescent red blood cells, and hepatocytes, considered to be an iron storage cell type. Importantly, communication between macrophages and hepatocytes exists whereby in certain conditions (e.g. inflammation) the former expresses pro-inflammatory cytokines such as Il-1 and Il-6 that activate transcription of hepcidin in the latter (57). In addition, others have observed that the resident liver macrophages (Kupffer cells) may be implicated in the setting of basal hepcidin levels through signaling to primary hepatocytes, as co-culturing the two cell types renders lower hepcidin levels than growing primary hepatocytes alone (58).

In the present work, we investigate the use of co-cultures of RAW 264.7 macrophages (59) and BNL CL.2 liver cells (60) (both having a Balb/c mouse background) as a surrogate system for primary Kupffer cells and hepatocytes. We start by establishing a dual IRP binding activity reporter, similar to that reported by Li *et al.* (61), where the first part (IG) is made by placing the Ferritin (Ft) IRE 5' to the enhanced green fluorescent protein (EGFP). The second part (TI) places the Transferrin Receptor (TfR) IRE 3' to tandem dimer (td)Tomato. Using these two parts, the ratio TI/IG inversely correlates with availability of iron levels. We use this reporter to detect an increase in IRP binding activity in BNL CL.2 cells when co-cultured with RAW 264.7. We further investigate this regulation by comparing the gene expression profiles of BNL CL.2 cells alone or treated with ferric ammonium citrate (FAC), in the

presence and absence of RAW 264.7. The resulting cell-line model system is a convenient platform for studying iron metabolism in a way that has only been possible previously with primary cells.

Materials and Methods

Plasmid preparation

Plasmids were made using the biobrick collection described in (62), and assembly was carried out as described previously. New parts (mouse ferritin (ft) and tranferrin receptor (TfR) IREs) were designed following the recommendations in the Biobricks Foundation Request For Comments (BBF RFC) 23. Briefly, the IREs were prepared either by use of oligonucleotide inserts (5'-CTAGACGCGGGTCTGTCTCTTGCTTCAACAGTGTTTGGACGGAACAGATCCGGGGACTAGTA GCGGCCGCTGCA-3') and 5'-GCGGCCGCTACTAGTCCCCGGATCTGTTCCGTCCAAACACTGTTGAAGCAAGAGACAGACCC GCGT-3') or by polymerase chain reaction (PCR) amplification using the primers 5'-CCTTTCTAGATTATATATAGAAGATAATTATC-3' and 5'-AAGGCTGCAGCGGCCGCTACTAGTACTGTTCCCGATAATTACGTAC-3'. Segments were then cloned into a biobrick cloning plasmid and, after assembly of the desired plasmids, the constructs were digested with XbaI and PstI and cloned into pEGFP-C1 (Clontech) digested with NheI and PstI, thereby removing the EGFP sequence and providing the CMV promoter upstream and polyA signal downstream of the biobrick insert.

Cell culture and transfection

HEK293, BNL CL.2 and RAW 264.7 cells were purchased from ATCC. Cells were propagated in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). RAW 264.7 conditioned medium was obtained by treating 1 $x10^6$ cells/ml with 100 ng/ml *Escherichia coli* serotype 055:B5 lipopolysacharide (LPS) (Sigma) in 86mm plates for 24h. For single culture experiments, cells were plated at $1.5x10^5$ cells/ml. Transfection was performed with lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, in 24-well plates: cells were incubated for 4 hours, with DNA and the lipofectamine reagent, after which new medium was added. After transfection, fresh medium, conditioned medium or fresh medium was added, with either 10 μM desferoxamine (DFO) or 100 μM ferric ammonium citrate (FAC), and cells were incubated for 24h. For co-culture experiments BNL CL.2 cells were plated at $7.5x10^4$ cells/ml on the bottom of the plate and a 0.4 μm pore insert was added (from Nunc for 24 well plates used for most experiments and from Becton Dickinson (BD) for 6-well plates used for microarray experiment), where an additional $7.5x10^4$ cells/ml of either BNL CL.2 or RAW 264.7 cells were plated in the insert. For the microarray experiment FAC was added to the co-culture at 100 μM, where appropriate.

Flow cytometry

Flow-assisted cell sorting (FACS) analysis was performed with FACSCanto (BD). Basal fluorescent levels were determined using untransfected cells. The average of fluorescence above background was taken for each experimental condition. To determine the ratio of tdTomato fluorescence over EGFP fluorescence, we first determined the fold-change of the fluorescent proteins for each treatment, as compared to control, and the ratio of the fold-changes was then determined.

RNA extraction and Real-time quantitative PCR analysis

Total RNA was extracted with the RNeasy kit (Qiagen). RT was performed with the Superscript II RT kit (Invitrogen), using random hexamers (Invitrogen). mRNA levels of β-actin and hepcidin were measured by real-time PCR in Lightcycler 480 (Roche) with the Lightcycler 480 SYBR Green I Master kit (Roche). The following primers were used: β-actin 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGTGTTGAAGGTCTCAAA-3'; hepcidin 5'-AGAGCTGCAGCCTTTGCAC-3' and 5'-GAAGATGCAGATGGGGAAGT-3'. Expression levels were normalized to the housekeeping gene β-actin.

Microarrays

RNA integrity was assessed with a Bioanalyzer 2100 (Agilent Technologies). Only samples with high integrity (RNA integrity number (RIN) >8) were subsequently used in microarray experiments.

Microarray expression profiles from these samples were obtained using the Affymetrix GeneChip® Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA according to the Affymetrix manuals GeneChip® 3' IVT Express Kit User Manual (P/N 702646 Rev.8) and Expression Wash, Stain and Scan User Manual (P/N 702731 Rev. 3). Stained arrays were scanned using an Affymetrix GeneChip® Scanner 3000 7G, generating CEL files.

Analysis was subsequently performed. Extracted log2-transformed intensities were normalized using the RMA (63) method to obtain summary expression values for each probe set. For determining differentially regulated probes, moderated paired t-tests were applied using limma (64). Probes with a p-value below 0.01 and additionally a fold change exceeding 1.4 in absolute value were selected to build the Venn diagrams.

Modeling

The behavior of the two reporter topologies proposed in the current work can be determined using the following system of ordinary differential equations:

$$\frac{dG}{dt} = \gamma_G \frac{1+f_1^{-1}(P/K_1)^n}{1+(P/K_1)^n} \frac{1+f_3^{-1}(T/K_3)^p}{1+(T/K_3)^p} - \delta_G G$$
$$\frac{dT}{dt} = \gamma_T \frac{f_2^{-1}+(P/K_2)^m}{1+(P/K_2)^m} \frac{1+f_4^{-1}(G/K_4)^q}{1+(G/K_4)^p} - \delta_T T$$

where the variables G,T and t denote the concentrations of IG and TI, respectively, and time. The parameter P is the concentration of IRP, and different values of this parameter distinguish between the cases referred to in Figure 1 as DFO, CTR and FAC. The parameters γ_G and γ_T represent the maximum rate of synthesis for the proteins IG and TI, respectively, while δ_G and δ_T, their degradation rates. The constants K_i represent the dissociation constants associated to the binding of the proteins on the mRNAs and the exponents n,m,p,q are the "Hill exponents". The factors f_i define the maximum fold change in translational activity. The equations above represent the reporter topology constructed in Figure 1, if $f_3=f_4=1$. A topology where IG inhibits TI and TI inhibits IG is achieved if $f_3,f_4 > 1$. The equations have been obtained following the textbook biochemical rate equation formalism. All parameters are estimates of the order of magnitude such that the solutions are comparable to the experimental data. We calculate the solution of these equations for the parameters values: K1=K2=50 nM, $K_3=K_4=1$ nM, $f_1=f_2=10$, $f_3=f_4=30$, n=1, m=p=q=2, γ=10 nM/min, δ=0.1 min^{-1}, IRP concentration in CTR case is 150 nM. This solution is represented as IG^* and TI^*, defined by the concentrations of IG and TI normalised to their values obtained at CTR.

Results

A dual reporter for IRP binding activity

In initial control experiments, using plasmids expressing EGFP or tdTomato without IREs, we observed a significant increase with DFO iron-chelator treatment (1.4-fold for EGFP and 1.3-fold for tdTomato) or reduction with FAC iron treatment (0.8-fold for both EGFP and tdTomato) (Fig 1a). This generic response was likely due to differences in the cell division times in the different treatments, since cell numbers after 48h increased with increasing iron (data not shown). Thus, the fluorescent proteins would be diluted when cells divided more rapidly, and more concentrated in slower-dividing cells, and were artifactually changing with iron concentration. To eliminate growth artifacts, we simply calculated ratios of tdTomato fluorescence over EGFP fluorescence (Fig 1a, inset). Consequently, these observations argued strongly in favor of developing a dual-fluorescent protein IRP reporter, as compared to one using a single fluorescent protein, to use similar ratios to avoid growth artifacts. We therefore engineered a dual-plasmid reporter with a plasmid containing the mouse transferring receptor IRE 3' to tdTomato, referred to as TI, and a second plasmid with the ferritin IRE 5' to EGFP, referred to as IG (see Figure 1b for plasmid schemas). Using this design, an increase in the ratio of TI/IG was expected to correlate with an increase in IRP binding activity.

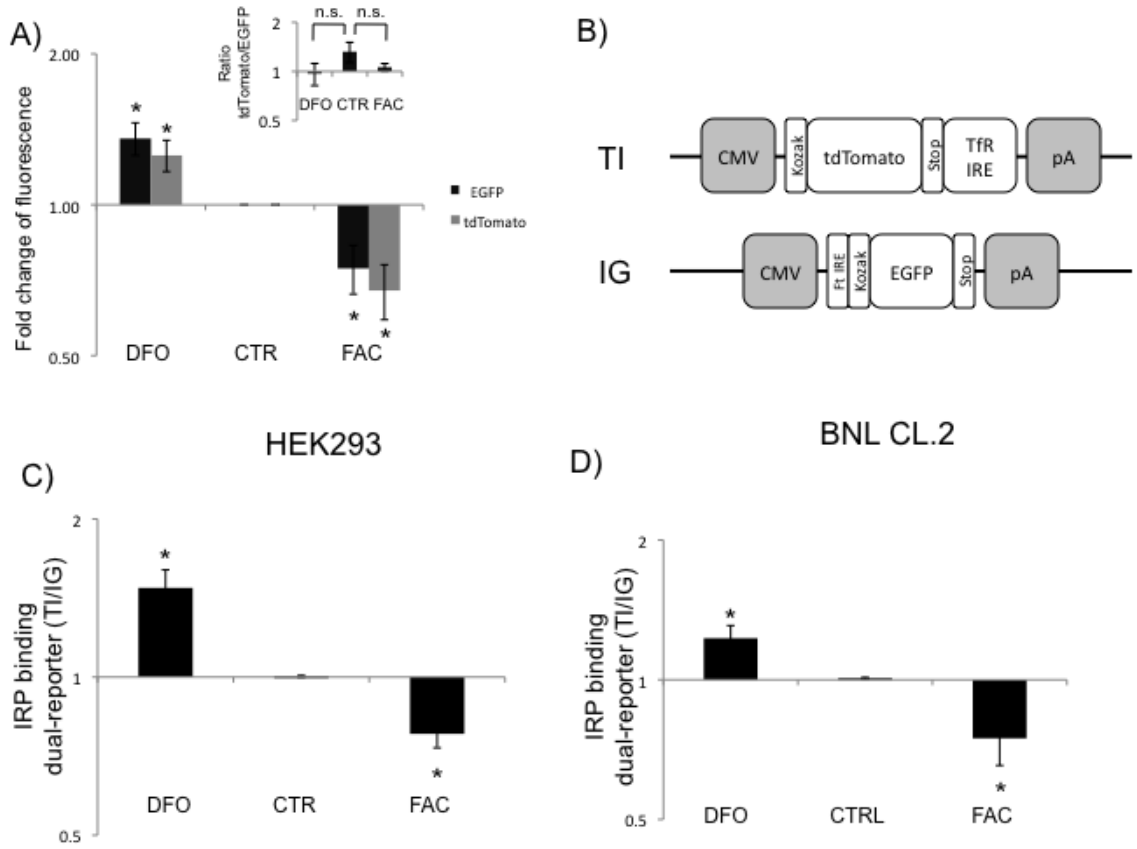

Figure 1. **Reporter of IRP binding activity.** (A) Fold change of fluorescence of control plasmids expressing EGFP or tandem dimer (td)Tomato when transfected into HEK293 cells, exhibiting significant (artifactual) changes after treatment with DFO or FAC for 48h, but not when the ratio of tdTomato/EGFP is taken (inset)(n=3). (B) TI and IG plasmids constitute the dual-reporter for IRP binding activity. (C) and (D) IRP binding activity of the dual-reporter in HEK 293 cells (C)(n=4) and in BNL CL.2 cells (D)(n=3). Cells were treated with DFO or FAC for 24h.*P<0.05 compared with control (CTR); n.s. - not significant.

To verify that our dual-reporter was behaving as expected we transiently transfected the plasmids in either HEK293 cells (Fig 1c) or BNL CL.2 (Fig 1d) and treated them with DFO or FAC. Indeed, the transfected cells modulated the levels of our IRP binding activity reporter by either increasing the ratio of TI/IG when the cells were treated with DFO (1.5-fold in HEK293 cells and 1.2-fold in BNL CL.2) or decreasing with FAC (0.8-fold in HEK 293 cells and 0.7-fold in BNL CL.2). Overall, the dual reporter behaved very reproducibly, giving high TI/IG signals in low iron concentrations and low (negative) signals in high iron concentrations.

Communication between RAW 264.7 and BNL CL.2 modulates IRP binding activity

Having evaluated the IRP binding activity reporter response to available free iron in the medium, we next investigated whether it was sensitive enough to detect signaled changes in IRP binding activity without directly changing the amount of extracellular iron. With this goal in mind, and to investigate the responses of BNL CL.2 liver cells to communication from RAW 264.7 macrophages, we treated BNL CL.2 liver cells (expressing the dual-reporter) with conditioned medium obtained from RAW 264.7 macrophages treated with *E. coli* LPS for 24h. When RAW 264.7 cells are treated with LPS, they release proinflammatory cytokines such as IL-6 and IL-1, and these modulate hepcidin expression and increase iron levels in hepatocytes (57). As expected, we found a decrease in the TI/IG ratio in BNL CL.2 cells treated with the conditioned medium, therefore indicating a decrease in IRP binding affinity by 0.6-fold (Figure 2a). In addition, we observed a concomitant increase in hepcidin mRNA expression (2-fold) by qRTPCR (Figure 2b).

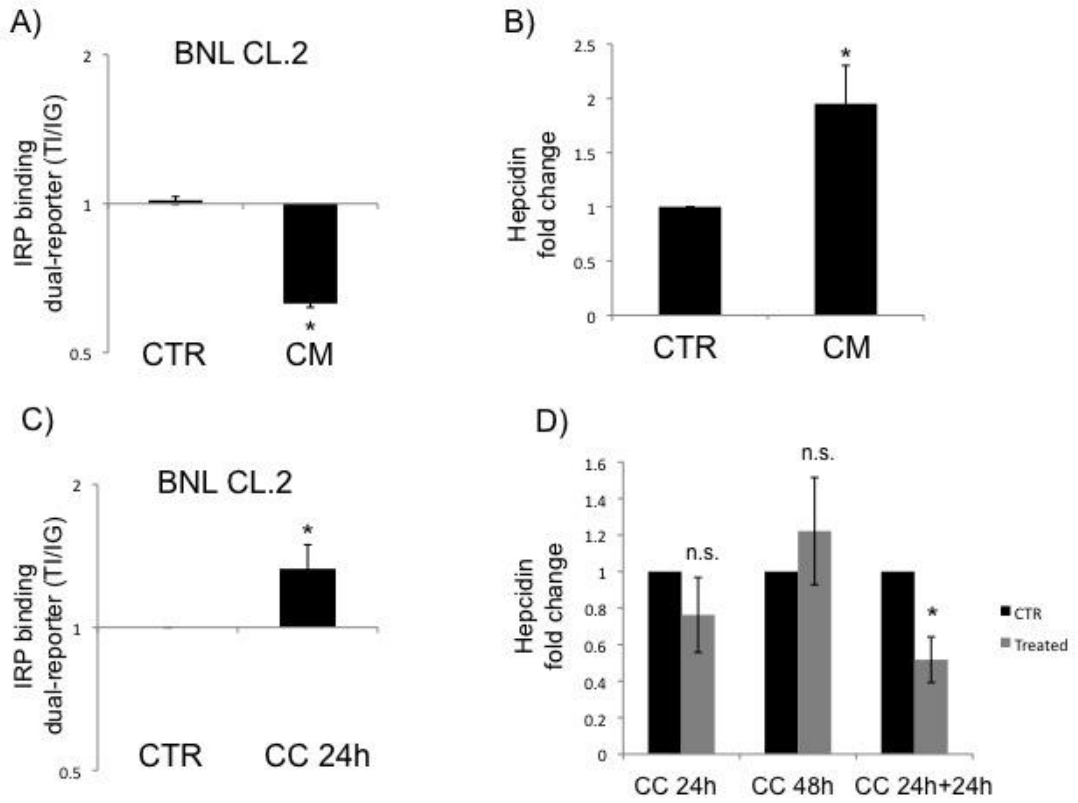

Figure 2. **Response of BNL CL.2 cells to conditioned medium and co-culture with RAW 264.7.** IRP Binding Activity (A) and hepcidin mRNA expression (B) as observed after 24h of treatment with conditioned medium (CM) of RAW 264.7 macrophages incubated with LPS (n=3). Response of the IRP binding dual-reporter (n=4)(C) and hepcidin expression (D) of cells in co-culture with RAW 264.7 for 24h (CC 24h)(n=6), for 48h (CC 48h)(n=4) or for 24h of co-culture followed by 24h of culture in the

same medium without the RAW 264.7 macrophages (CC 24h+24h)(n=4). *P<0.05 compared with control (CTR); n.s. - not significant.

Others have recently reported that co-culture of primary Kupffer cells and hepatocytes inhibits hepcidin expression (58). Similarly, we were interested in using RAW 264.7 and BNL CL.2 co-cultures as a surrogate for Kupffer cells and hepatocytes. Interestingly, 24h after co-culturing of these two cell types we observed an increased in IRP binding activity (1.3-fold), indicating that communication between these two cell types produce measurable effects with our dual-reporter. However, although an apparently concomitant decrease of 0.8-fold in hepcidin expression was observed, the difference obtained was not statistically significant (measured by qRT-PCR on BNL CL.2 cells after 24h of co-culture with RAW 264.7 macrophages; Figure 2d).

We hypothesized that longer times of co-culture of the two cell types might increase the difference of hepcidin described by others (58). We therefore measured hepcidin levels by qRT-PCR after 48h in co-culture but again found no significant drop; in fact, an apparent increase of hepcidin was observed (1.2-fold). To investigate whether there was a compensatory effect from RAW 264.7 cells with long culture times, we grew BNL CL.2 cells for 24h in co-culture, after which time the RAW 264.7 macrophages were removed and BNL CL.2 cells were cultured in the same medium for an additional 24h. In these conditions, there was indeed a statistically significant decrease in hepcidin expression (0.6 fold). Taken together these data suggest that the decrease in hepcidin expression observed here in BNL CL.2-RAW 264.7 co-cultures, and by others in Kupffer cell and hepatocyte primary co-cultures, may depend on the specific experimental conditions used and, possibly, the macrophage gene network status.

BNL CL.2 gene network expression profiling after RAW 264.7 co-culture using microarrays

After observing the modulation in IRP binding activity in the above BNL CL.2 and RAW 264.7 co-cultures, we were interested in screening which genes might be implicated in this communication, from the standpoint of the BNL CL.2 cells. We therefore performed microarray analysis in BNL CL.2 that were cultured for 24h with a cell culture insert also plated with BNL CL.2 (BB), the same setup with FAC (FBB), BNL CL.2 with RAW 264.7 in the cell culture insert (BR), or the latter setup with added FAC (FBR).

It should be noted that we observed a high variation of gene expression from experiment to experiment for each group, suggesting that the status of BNL CL.2 cells varies considerably with time in culture (data not shown). In spite of this high variation, we found several genes (132) that consistently varied between each treatment, as compared with BNL CL.2 grown alone and without treatment as a control. In this set of 132 differentially expressed genes, we found that the treatment with BNL CL.2 + RAW 264.7 + FAC (FBR) accounted for most of the differences found (115/132), as is shown in Figure 3. The FBR treatment not only covers large parts of the FBB (32/44 genes) and BR (27/33 genes) treatments, as might be expected, but the presence of FAC, together with the co-culturing with RAW 264.7 (the FBR set), seems to have a synergistic effect on the BNL CL.2 cells: an additional 57 differentially expressed genes are found that are not present in either the FBB or the BR sets (See Supplementary Data 1 for the list of differentially expressed genes). In the list of modulated genes we find several that have been previously implicated in iron metabolism such as the iron importer transferring receptor (TfR), the ferroxidase

ceruloplasmin (Cp) or the signal transducer and activator of transcription 1 (stat1) (65). Interestingly, we have found several interferon-related genes such as the interferon-activated gene (Ifi) 202b and 203 or the interferon-induced protein 35 suggesting that RAW 264.7 macrophage-produced interferon may be modulating the basal levels of IRP binding activity in BNL CL.2 cells.

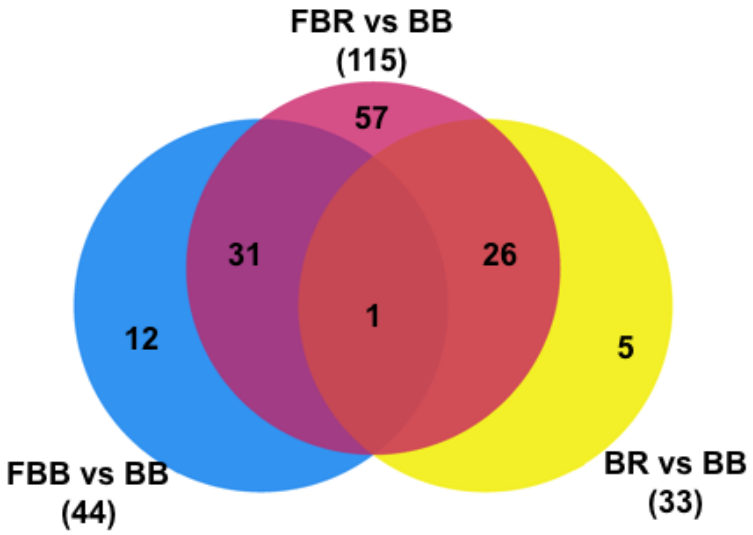

Figure 3. **Venn Diagram of differentially expressed genes.** BNL CL.2 cells alone (BB) were used as control for comparisons of BNL CL.2 treated with FAC (FBB), co-cultured with RAW 264.7 macrophages (BR) or both co-cultured with RAW 264.7 and treated with FAC (FBR).

Analyzing the obtained results using GOEast (66) as a gene ontology (GO) enrichment tool, and setting a minimum of a p-value of 0.01, we found several GO terms associated with the immune system (see table 1). Our model system of BNL CL.2 and RAW 264.7 co-culture therefore suggests that Kupffer cells exert a regulation of the immune-related genes in hepatocytes.

Table 1 – Gene ontology enrichment of all differentially expressed genes.

GOID	Term	Probes	p Value
GO:0006955	immune response	21	<0.0001
GO:0002376	immune system process	28	<0.0001
GO:0042221	response to chemical stimulus	38	<0.0001
GO:0006952	defense response	19	<0.0001
GO:0006954	inflammatory response	14	<0.0001
GO:0070887	cellular response to chemical stimulus	18	<0.0001
GO:0006695	cholesterol biosynthetic process	7	<0.0001
GO:0016126	sterol biosynthetic process	7	<0.0001
GO:0050896	response to stimulus	58	0.0001
GO:0008299	isoprenoid biosynthetic process	5	0.0008
GO:0009611	response to wounding	15	0.0008
GO:0006694	steroid biosynthetic process	7	0.0012
GO:0003166	bundle of His development	3	0.0012
GO:0003164	His-Purkinje system development	3	0.0012
GO:0003161	cardiac conduction system development	3	0.0020
GO:0010035	response to inorganic substance	11	0.0026
GO:0008203	cholesterol metabolic process	7	0.0028
GO:0006950	response to stress	29	0.0028
GO:0016125	sterol metabolic process	7	0.0041
GO:0050900	leukocyte migration	6	0.0044
GO:0002682	regulation of immune system process	14	0.0045
GO:0045651	positive regulation of macrophage differentiation	3	0.0056
GO:0043433	negative regulation of sequence-specific DNA binding transcription factor activity	5	0.0063
GO:0002684	positive regulation of immune system process	11	0.0085
GO:0050901	leukocyte tethering or rolling	3	0.0087
GO:0045649	regulation of macrophage differentiation	3	0.0087

Discussion

We engineered a dual-reporter system of IRP binding activity that directly correlates with available iron levels in the medium, as seen by chelating iron with DFO or through FAC iron addition. Both treatments induced or repressed the expression of TI and IG in such a way that the ratio TI/IG always changed by over 20%, in the experimental conditions tested. Others have previously reported an IRP binding activity reporter (61). However, in that study, the authors first depleted the cells of iron by growing them in medium without serum (with 0.2 μM of iron). This induces a large change in the reporter after addition of different sources of iron, but cannot be compared to the results described here since we were interested in conditions where serum was always present, to be closer to physiological conditions .

Our reporter, as described, was sensitive enough to detect significant changes in response to DFO and FAC, as compared to controls, even in the presence of serum. However, it is still possible to envision situations where a more sensitive reporter might be of interest. Such a reporter could in principle be engineered by improving the topology of the network through the implementation of negative regulations from TI to IG and from IG to TI (see Figure 4a). Analyzing this model computationally (see Methods),

we found the solution included in Figure 4b, which indicates that a slightly more sensitive response could be achieved with mutually-inhibitory reporter network connections.

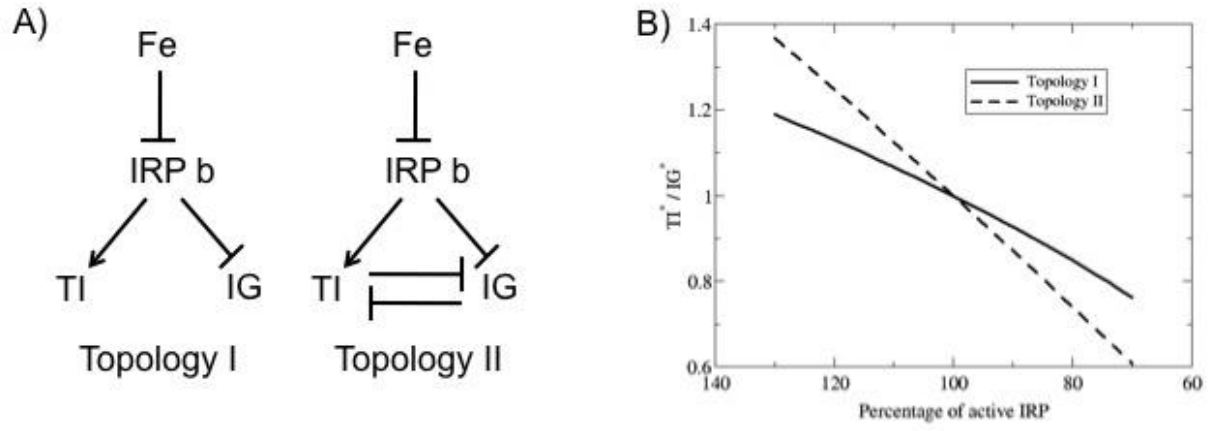

Figure 4. **Modelling of dual-fluorescence IRP binding activity reporter.** (A) Topologies used for modeling, Fe – Iron; IRPb – binding IRP. Topology I was the reporter constructed in this study. Topology II is a theoretical improvement with extra inhibitory connections. (B) Curves of the ratios of TI/IG for the topologies in (A) with varying binding IRP.

Although there is the potential to improve network topologies, the dual-reporter engineered here was sufficiently sensitive to detect reproducibly the modulation of IRP binding activity in BNL CL.2, both in response to conditioned medium from RAW 264.7 macrophages treated with LPS and in response to co-culture with RAW 264.7 macrophages. To our knowledge, this is the first time that it has been shown that this type of reporter can be significantly modulated by a stimulus other than addition or removal of the available iron in the medium.

In addition, we believe that this is the first report where RAW 264.7 and BNL CL.2 have been co-cultured to study communication between these two cell types. Interestingly, BNL CL.2 seems to conserve the pathways for hepcidin regulation not only in response to the conditioned medium (67), but also in response to co-culturing with macrophages (58).

To further investigated which pathways may be implicated in the modulation of the IRP binding activity when co-culturing BNL CL.2 and RAW 264.7 cells we analyzed the gene expression profile in BNL CL.2, arising from the communication with RAW 264.7 macrophages, using microarray analysis. Macrophages are an important component of the innate immune system and it is not surprising that many genes we found regulated in our experimental conditions also modulate the basal expression levels of genes that are implicated in the immune system, as observed by the gene enrichment analysis. This seems to suggest that the pathways of innate immunity (e.g. interferons) may be implicated in setting the basal levels of IRP binding activity, which will ultimately affect cellular iron homeostasis.

In summary, we have developed a dual reporter of IRP activity that responds to cues in the extracellular environment and we demonstrate its utility by studying the response of IRP activity to co-culture of BNL CL.2 liver cells with RAW 264.7 macrophages. We investigate which genes are modulated in this co-culture system, providing a platform for addressing how macrophages may participate in the iron homeostasis of liver cells and, ultimately, in systemic iron metabolism. The work with cell lines instead of primary cultures opens the way to applying many other molecular biology techniques and improves significantly on the time- and price-constraints of experiments.

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Acknowledgments

We would like to thank Jennifer Semple for critical reading of the manuscript.

Supplementary Data 1 – List of differentially expressed genes

ID	Gene.Symbol	FC_HR_HH	P.Val_HR_HH	FC_FHH_HH	P.Val_FHH_HH	FC_FHR_HH	P.Val_FHR_HH
1443858_at	**9230105E10Rik**	**1.7699**	0.0065	**1.0732**	0.2867	**1.7569**	0.0096
1456643_at	**9230114K14Rik**	**-1.2431**	0.0194	**-1.4164**	0.0045	**-1.3362**	0.0055
1436172_at	**9530028C05**	**1.8608**	0.0079	**1.1721**	0.0493	**2.0059**	0.0014
1436825_a_at	**A630095E13Rik**	**-1.1411**	0.0406	**1.4941**	0.0007	**1.4153**	0.0010
1450095_a_at	**Acyp1**	**-1.0282**	0.6093	**-1.1877**	0.1071	**-1.4094**	0.0097
1421172_at	**Adam12**	**1.0724**	0.1521	**-1.3296**	0.0014	**-1.4639**	0.0002
1439276_at	**Adar**	**1.5611**	0.0112	**1.1682**	0.0254	**1.6290**	0.0001
1425405_a_at	**Adar**	**1.3514**	0.0124	**1.0870**	0.1613	**1.7114**	0.0000
1425220_x_at	**AF067061**	**-1.3215**	0.0887	**1.4130**	0.0010	**1.2532**	0.0462
1416645_a_at	**Afp**	**1.0684**	0.1970	**1.3778**	0.0004	**1.4448**	0.0016
1418752_at	**Aldh3a1**	**-1.1953**	0.0201	**1.5020**	0.0019	**1.4888**	0.0003
1424785_at	**Angptl6**	**-1.0048**	0.9315	**1.1626**	0.0849	**1.4654**	0.0075
1426418_at	**Atoh8**	**-1.1803**	0.0869	**-1.1830**	0.0069	**-1.4519**	0.0006
1434372_at	**AW112010**	**1.1809**	0.0088	**1.1642**	0.0270	**1.4372**	0.0003
1435454_a_at	**BC006779**	**1.7321**	0.0101	**-1.0037**	0.9612	**1.8237**	0.0023
1445849_at	**BC080696**	**-1.1014**	0.1359	**1.5680**	0.0021	**1.6072**	0.0086
1451386_at	**Blvrb**	**-1.0143**	0.7938	**1.4924**	0.0001	**1.5161**	0.0001
1424921_at	**Bst2**	**1.3387**	0.0197	**1.1164**	0.0556	**1.5359**	0.0002
1417009_at	**C1ra**	**1.1935**	0.0622	**1.1012**	0.0881	**1.4137**	0.0007
1424041_s_at	**C1s**	**1.2418**	0.0542	**1.1323**	0.0309	**1.6044**	0.0009
1423954_at	**C3**	**-1.1002**	0.0882	**1.2386**	0.0267	**1.4937**	0.0001
1453233_s_at	**Calr3**	**-1.0941**	0.0639	**1.5052**	0.0003	**1.3904**	0.0022
1431255_at	**Calr3**	**-1.1371**	0.0508	**1.5091**	0.0000	**1.4857**	0.0035
1453232_at	**Calr3**	**-1.0495**	0.2879	**1.6048**	0.0000	**1.6843**	0.0007
1420380_at	**Ccl2**	**1.5309**	0.0081	**1.0809**	0.1340	**2.0137**	0.0001
1422029_at	**Ccl20**	**1.2665**	0.0074	**1.6168**	0.0017	**2.4490**	0.0002
1421228_at	**Ccl7**	**1.4068**	0.0037	**-1.0667**	0.1871	**1.6170**	0.0004
1417496_at	**Cp**	**-1.1050**	0.1027	**1.2242**	0.0407	**1.4016**	0.0006
1455393_at	**Cp**	**-1.0455**	0.3527	**1.2717**	0.0047	**1.5834**	0.0001
1426243_at	**Cth**	**1.0973**	0.1461	**1.3439**	0.0019	**1.4292**	0.0029
1457644_s_at	**Cxcl1**	**1.1836**	0.0073	**1.0637**	0.2425	**1.4688**	0.0001
1419209_at	**Cxcl1**	**1.1698**	0.0373	**1.0766**	0.1625	**1.5014**	0.0001
1449984_at	**Cxcl2**	**1.3187**	0.0014	**1.0045**	0.9502	**1.4910**	0.0036
1438148_at	**Cxcl3**	**1.2102**	0.0746	**1.0469**	0.5755	**1.9236**	0.0024
1460011_at	**Cyp26b1**	**-1.3213**	0.0175	**-1.4266**	0.0002	**-1.6093**	0.0005
1450646_at	**Cyp51**	**1.0358**	0.5982	**1.4427**	0.0028	**1.3796**	0.0039
1456890_at	**Ddx58**	**1.7654**	0.0097	**1.1540**	0.0179	**2.1502**	0.0023
1436562_at	**Ddx58**	**1.9294**	0.0042	**1.0649**	0.2476	**2.3844**	0.0000
1428781_at	**Dmkn**	**-1.4326**	0.0008	**1.0215**	0.7613	**-1.2407**	0.0145
1416552_at	**Dppa5a**	**-1.0119**	0.8490	**1.5108**	0.0001	**1.3940**	0.0005
1419639_at	**Efnb2**	**-1.1373**	0.0790	**-1.2163**	0.0052	**-1.4101**	0.0003
1417065_at	**Egr1**	**-1.2424**	0.0033	**-1.4656**	0.0015	**-1.6139**	0.0001
1436329_at	**Egr3**	**-1.2382**	0.1590	**-1.5629**	0.0003	**-1.7789**	0.0000
1440866_at	**Eif2ak2**	**1.3389**	0.0143	**1.0113**	0.8145	**1.4179**	0.0082
1448136_at	**Enpp2**	**1.2922**	0.0147	**1.3142**	0.0012	**1.8749**	0.0001
1417382_at	**Entpd5**	**-1.1459**	0.0537	**1.4359**	0.0007	**1.2628**	0.0088

ID	Gene.Symbol	FC_HR_HH	P.Val_HR_HH	FC_FHH_HH	P.Val_FHH_HH	FC_FHR_HH	P.Val_FHR_HH
1448798_at	**Eps8l3**	**-1.0041**	0.9470	**1.0210**	0.7604	**1.5235**	0.0009
1438322_x_at	**Fdft1**	**1.0810**	0.1953	**1.4518**	0.0021	**1.3781**	0.0047
1423418_at	**Fdps**	**1.0771**	0.2754	**1.4399**	0.0013	**1.4297**	0.0018
1419086_at	**Fgfbp1**	**1.0149**	0.7909	**1.3924**	0.0006	**1.5364**	0.0001
1449519_at	**Gadd45a**	**1.0223**	0.6420	**1.3594**	0.0030	**1.4124**	0.0062
1453851_a_at	**Gadd45g**	**-1.0764**	0.1689	**-1.3020**	0.0570	**-1.4453**	0.0040
1457472_at	**Gigyf2**	**-1.0716**	0.3497	**-1.3504**	0.0097	**-1.5706**	0.0055
1421041_s_at	**Gm3776 /// Gsta1 /// Gsta2**	**-1.0257**	0.5491	**1.4774**	0.0029	**1.4776**	0.0015
1420394_s_at	**Gp49a /// Lilrb4**	**1.4981**	0.0075	**1.0700**	0.3869	**1.4042**	0.0023
1421040_a_at	**Gsta2**	**1.0574**	0.3452	**2.0792**	0.0005	**1.9973**	0.0003
1429184_at	**Gvin1**	**1.6514**	0.0207	**-1.1276**	0.0773	**1.6604**	0.0098
1425614_x_at	**H2-D1**	**1.1313**	0.1602	**1.0813**	0.1257	**1.4115**	0.0036
1451644_a_at	**H2-gs10 /// H2-Q6 /// LOC68395**	**1.2315**	0.0045	**1.1090**	0.1680	**1.4603**	0.0011
1427746_x_at	**H2-K1**	**1.1222**	0.1433	**1.0940**	0.1334	**1.4094**	0.0035
1441536_at	**Hmgcs1**	**-1.0157**	0.7996	**1.4173**	0.0029	**1.3206**	0.0057
1448239_at	**Hmox1**	**1.0977**	0.3187	**1.4115**	0.0004	**1.5441**	0.0009
1453596_at	**Id2**	**-1.3688**	0.0455	**-1.1077**	0.1340	**-1.6764**	0.0045
1422537_a_at	**Id2**	**-1.3328**	0.0116	**-1.1876**	0.0229	**-1.5763**	0.0030
1435176_a_at	**Id2**	**-1.2752**	0.0288	**-1.1729**	0.0981	**-1.5405**	0.0028
1416630_at	**Id3**	**-1.1186**	0.0923	**-1.2718**	0.0009	**-1.5526**	0.0004
1422433_s_at	**Idh1**	**-1.0272**	0.5075	**1.4124**	0.0006	**1.2956**	0.0056
1451122_at	**Idi1**	**1.0458**	0.4414	**1.4652**	0.0011	**1.3769**	0.0016
1455881_at	**Ier5l**	**-1.1265**	0.1543	**-1.4051**	0.0001	**-1.3925**	0.0002
1457666_s_at	**Ifi202b**	**2.0400**	0.0028	**1.3731**	0.0005	**2.6099**	0.0000
1421551_s_at	**Ifi202b**	**1.8616**	0.0132	**1.2615**	0.0076	**2.4762**	0.0000
1448775_at	**Ifi203**	**2.2648**	0.0042	**1.1528**	0.0165	**3.0943**	0.0001
1451567_a_at	**Ifi203 /// LOC100044071**	**3.9416**	0.0030	**1.3881**	0.0008	**5.0349**	0.0000
1452348_s_at	**Ifi204 /// Ifi205 /// Mnda /// Mndal**	**2.1059**	0.0060	**1.2420**	0.0075	**2.1283**	0.0011
1452349_x_at	**Ifi205 /// Mnda**	**1.4665**	0.0057	**-1.1154**	0.0995	**1.3387**	0.0034
1459151_x_at	**Ifi35**	**1.4031**	0.0086	**1.0454**	0.3740	**1.5453**	0.0004
1445897_s_at	**Ifi35**	**1.3074**	0.0324	**1.0170**	0.7191	**1.5114**	0.0006
1424617_at	**Ifi35**	**1.3830**	0.0107	**1.0991**	0.0801	**1.5750**	0.0002
1426276_at	**Ifih1**	**1.8745**	0.0025	**1.0058**	0.9189	**1.9602**	0.0007
1417141_at	**Igtp**	**1.6584**	0.0049	**-1.0612**	0.2665	**1.8236**	0.0003
1425958_at	**Il1f9**	**1.2802**	0.0187	**1.2075**	0.0443	**1.5948**	0.0008
1426181_a_at	**Il24**	**1.0762**	0.1000	**-1.4512**	0.0014	**-1.2441**	0.0861
1454671_at	**Insig1**	**-1.0236**	0.6535	**1.5989**	0.0007	**1.4349**	0.0041
1417244_a_at	**Irf7**	**1.2282**	0.0145	**1.0975**	0.1944	**1.5524**	0.0001
1421322_a_at	**Irf9**	**2.4878**	0.0007	**-1.0324**	0.6031	**2.7324**	0.0000
1418825_at	**Irgm1**	**1.4132**	0.0098	**1.0225**	0.6340	**1.6193**	0.0004
1417793_at	**Irgm2**	**1.6473**	0.0110	**1.1373**	0.1802	**1.9697**	0.0015
1439878_at	**Ivl**	**-1.1218**	0.0710	**-1.4065**	0.0135	**-1.5887**	0.0049
1452707_at	**Klhl30**	**1.0739**	0.1398	**1.3301**	0.0006	**1.4195**	0.0003
1437268_at	**Lancl3**	**1.1371**	0.1173	**1.3812**	0.0325	**1.4816**	0.0039
1420741_x_at	**Lce1i**	**1.1726**	0.0058	**-1.5867**	0.0001	**-1.5171**	0.0005
1427747_a_at	**Lcn2**	**1.0516**	0.5178	**1.3380**	0.0007	**1.8875**	0.0032
1436317_at	**LOC100504348**	**-1.1801**	0.0227	**-1.1365**	0.1214	**-1.4318**	0.0092
1418288_at	**Lpin1**	**-1.0308**	0.4614	**1.4202**	0.0059	**1.4500**	0.0015
1420013_s_at	**Lss**	**1.0821**	0.2419	**1.4420**	0.0015	**1.5333**	0.0001
1438930_s_at	**Mecp2**	**-1.1445**	0.0153	**-1.2363**	0.0139	**-1.4071**	0.0005

ID	Gene.Symbol	FC_HR_HH	P.Val_HR_HH	FC_FHH_HH	P.Val_FHH_HH	FC_FHR_HH	P.Val_FHR_HH
1420450_at	**Mmp10**	**1.2943**	0.0506	**1.1441**	0.1315	**1.6594**	0.0000
1417256_at	**Mmp13**	**1.3842**	0.0139	**1.1744**	0.0430	**1.6503**	0.0006
1452231_x_at	**Mndal**	**2.3350**	0.0011	**1.1210**	0.0810	**2.8115**	0.0000
1426906_at	**Mndal**	**2.6139**	0.0079	**1.1595**	0.1386	**3.4513**	0.0003
1448663_s_at	**Mvd**	**-1.0349**	0.6276	**1.5037**	0.0034	**1.5375**	0.0008
1417303_at	**Mvd**	**1.1590**	0.0760	**1.3811**	0.0008	**1.4422**	0.0005
1425923_at	**Mycn**	**2.1156**	0.0095	**1.0616**	0.2693	**1.3613**	0.0004
1425344_at	**Narf**	**-1.1488**	0.3270	**-1.6127**	0.0000	**-1.7030**	0.0000
1439556_at	**Ncam1**	**-1.2041**	0.0304	**-1.2577**	0.0045	**-1.4254**	0.0015
1441305_at	**Nedd4l**	**-1.1516**	0.0987	**-1.2945**	0.0038	**-1.5767**	0.0020
1458299_s_at	**Nfkbie**	**1.1749**	0.0944	**1.0082**	0.9078	**1.5287**	0.0059
1425719_a_at	**Nmi**	**1.3380**	0.0079	**1.1011**	0.1184	**1.5245**	0.0002
1423627_at	**Nqo1**	**-1.0525**	0.2868	**1.4358**	0.0010	**1.3915**	0.0015
1418355_at	**Nucb2**	**-1.0223**	0.5969	**1.3238**	0.0061	**1.4667**	0.0008
1424775_at	**Oas1a**	**1.5973**	0.0090	**1.0152**	0.7619	**1.6380**	0.0012
1418674_at	**Osmr**	**1.1940**	0.0140	**1.1328**	0.1006	**1.7069**	0.0008
1419853_a_at	**P2rx7**	**1.0611**	0.5971	**-1.4676**	0.0095	**-1.4532**	0.0083
1426774_at	**Parp12**	**1.5073**	0.0038	**1.0579**	0.3536	**1.7125**	0.0004
1451564_at	**Parp14**	**1.7236**	0.0036	**-1.0671**	0.2091	**1.8271**	0.0039
1416897_at	**Parp9**	**1.4346**	0.0051	**1.0150**	0.7999	**1.6922**	0.0004
1418480_at	**Ppbp**	**-1.0522**	0.2718	**-1.4715**	0.0003	**-1.4622**	0.0003
1449824_at	**Prg4**	**1.2988**	0.0016	**1.4298**	0.0008	**1.6683**	0.0000
1417856_at	**Relb**	**1.2015**	0.0275	**1.0775**	0.3506	**1.4577**	0.0070
1418580_at	**Rtp4**	**4.5077**	0.0043	**1.0849**	0.1078	**5.8963**	0.0002
1450826_a_at	**Saa3**	**1.5379**	0.0000	**1.5725**	0.0000	**2.1318**	0.0001
1460603_at	**Samd9l**	**1.6404**	0.0055	**1.0023**	0.9694	**1.6788**	0.0023
1418206_at	**Sdf2l1**	**-1.0629**	0.2568	**1.5073**	0.0004	**1.4684**	0.0010
1440173_x_at	**Selp**	**1.0760**	0.2070	**-1.4320**	0.0068	**-1.8257**	0.0001
1420558_at	**Selp**	**1.1382**	0.0452	**-1.4156**	0.0009	**-1.4448**	0.0010
1449906_at	**Selp**	**1.0623**	0.4709	**-1.4081**	0.0021	**-1.3867**	0.0350
1426318_at	**Serpinb1b**	**1.0984**	0.0573	**1.5708**	0.0000	**1.4874**	0.0002
1451139_at	**Slc39a4**	**1.1322**	0.0210	**1.2430**	0.0016	**1.6059**	0.0007
1450409_a_at	**Slc48a1**	**-1.0106**	0.8422	**1.3261**	0.0011	**1.4544**	0.0001
1450410_a_at	**Slc48a1**	**1.0236**	0.5913	**1.3940**	0.0002	**1.5349**	0.0001
1417636_at	**Slc6a9**	**1.0763**	0.3047	**1.2840**	0.0025	**1.4147**	0.0005
1443536_at	**Slc7a11**	**1.1495**	0.3003	**1.5005**	0.0086	**1.3956**	0.0099
1420413_at	**Slc7a11**	**1.1076**	0.0485	**1.7835**	0.0001	**2.1445**	0.0000
1448377_at	**Slpi**	**1.2418**	0.1229	**1.3806**	0.0002	**1.7659**	0.0000
1420562_at	**Slurp1**	**-1.4779**	0.0016	**1.0901**	0.2474	**-1.1463**	0.1553
1428471_at	**Sorbs1**	**-1.0667**	0.1576	**-1.3113**	0.0032	**-1.4054**	0.0034
1422672_at	**Sprr1b**	**-1.0867**	0.1572	**-1.5832**	0.0002	**-1.4755**	0.0026
1422963_at	**Sprr2i**	**-1.0377**	0.3841	**-1.4233**	0.0018	**-1.5375**	0.0020
1450034_at	**Stat1**	**1.4267**	0.0082	**-1.0592**	0.2368	**1.4922**	0.0045
1440481_at	**Stat1**	**1.4291**	0.0336	**1.1699**	0.0265	**1.5142**	0.0015
1450033_a_at	**Stat1**	**1.3838**	0.0221	**-1.0359**	0.4937	**1.6810**	0.0027
1420729_at	**Tcstv1**	**-1.4931**	0.0001	**1.2632**	0.0710	**1.1814**	0.2098
1422967_a_at	**Tfrc**	**-1.1411**	0.1147	**-2.5243**	0.0000	**-2.6780**	0.0001
1452661_at	**Tfrc**	**-1.0096**	0.8482	**-2.3741**	0.0000	**-2.3166**	0.0000
1419132_at	**Tlr2**	**1.2227**	0.0109	**1.1393**	0.2440	**1.4136**	0.0065
1426065_a_at	**Trib3**	**1.1057**	0.1069	**1.4123**	0.0025	**1.5052**	0.0023

ID	Gene.Symbol	FC_HR_HH	P.Val_HR_HH	FC_FHH_HH	P.Val_FHH_HH	FC_FHR_HH	P.Val_FHR_HH
1456225_x_at	**Trib3**	**1.1020**	0.1562	**1.4666**	0.0018	**1.4424**	0.0027
1436199_at	**Trim14**	**1.3205**	0.0060	**-1.0309**	0.5190	**1.4205**	0.0008
1418077_at	**Trim21**	**1.4007**	0.0016	**1.1243**	0.0439	**1.3011**	0.0031
1448940_at	**Trim21**	**1.6663**	0.0001	**1.0785**	0.1423	**1.6445**	0.0003
1427960_at	**Ugt2b34**	**-1.2720**	0.0227	**1.6992**	0.0001	**1.7172**	0.0010
1427961_s_at	**Ugt2b34**	**-1.1160**	0.3659	**1.7514**	0.0012	**1.6078**	0.0024
1418191_at	**Usp18**	**2.5367**	0.0067	**-1.1203**	0.0532	**2.6764**	0.0024
1448162_at	**Vcam1**	**1.3713**	0.0205	**1.3113**	0.0219	**2.1045**	0.0062
1443698_at	**Xaf1**	**5.0180**	0.0011	**1.0578**	0.6496	**6.8893**	0.0000

4. SUPPLEMENTARY ARTICLE 1

BUILDING BLOCKS FOR PROTEIN INTERACTION DEVICES

My contribution to Supplementary Article 1 consisted in the participation in the development of the semi-automated streamlined version for the Biobrick assembly protocol. I established three solution mixes with the required cocktails of enzymes in the corresponding buffers to digest the upstream part, the downstream part and the recipient vector (although in the paper a PCR is used to obtain the recipient vector instead of a digestion). I confirmed that the solutions with the mix of enzymes resisted freezing, repeated freeze-thaw cycles and conservation at -20 °C for several months in regards to the activity of the enzymes.

Grünberg R, Ferrar TS, van der Sloot AM, Constante M, Serrano L. Building blocks for protein interaction devices. Nucleic Acids Res. 2010 May;38(8):2645-62.

5. SUPPLEMENTARY ARTICLE 2

AVOIDING TRANSCRIPTION FACTOR COMPETITION AT PROMOTER LEVELS INCREASES THE CHANCES OF OBTAINING OSCILLATION

My contribution to Supplementary Article 2 consisted in the participation in the experimental design and analysis of the modeled data. The paper was the result of a series of analyses done to investigate the importance of the parameter values and network topologies to engineer an RNA-based oscillator. We intended to use a translation activator based on the artificial recruitment of the 40S ribosome subunit by a MS2-based tethering of the eukaryotic initiation factor 4E (eif4E), to the middle of the RNA, resulting in the expression of a second cistron, similar to work presented by others (68). Unfortunately, we did not detect changes in the expression of the reporter present in the second cistron (EGFP). This might have been due to the use of MS2 – which as a dimer may result in steric hindrance of the initiation factors – instead of the lambda N peptide (a monomer) as used in the paper (68). MS2 was used because the modeling suggested a monomer would not have a sufficiently steep activation curve, relative to levels of the eif4E activator, for the desired oscillations to occur. Alternatively, activation may have taken place but a more sensitive reporter would have been needed to observe it, such as luciferase instead of EGFP, as used in the original paper (68). We did not have the opportunity to follow up on the experiments and the Supplementary Article 2 was published with part of the theoretical analysis that was performed.

Munteanu A, Constante M, Isalan M, Solé RV. Avoiding transcription factor competition at promoter level increases the chances of obtaining oscillation. BMC Syst Biol. 2010 May 17;4:66.

6. Conclusion and Future Perspectives

6.1 Article 1

A biobrick library for cloning custom eukaryotic plasmids.

In this article we prepared a set of Biobricks in a format that allows for protein fusions. In our experiments, and in discussions with other potential users in our institute, we realized that the preparation of Biobricks for our proteins of interest is not always practical. We therefore created a set of Multiple Cloning Sites (MCSs) to allow the combination of the Biobrick assembly system and 'classical' cloning. We chose to present the new Biobricks as stand-alone collection to make it easier for the non-synthetic biologists to access it. We have prepared the **Article 1** as an instruction manual as to how to use the collection and illustrate the use of the library with a few examples, including recombinase-mediated cassette insertion for making stable cell lines by gene exchange.

Several other biobricks were prepared for our projects that are not present in this collection. Specifically, we were designing tools to control post-transcriptional parameters (see figure 6.1 for the list of parameters). The parts were ultimately to be used in engineering oscillators of the type described in **Supplementary Article 2**.

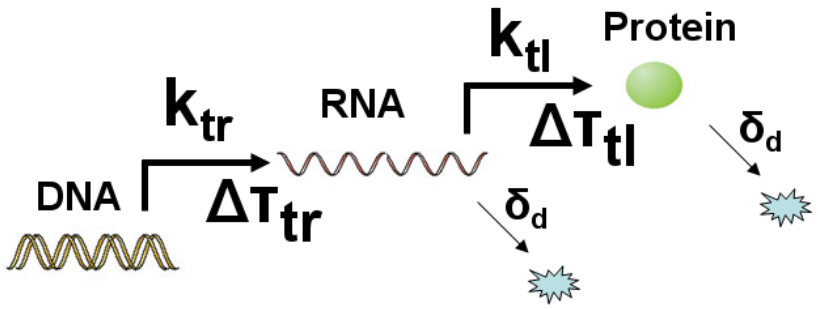

Figure 4.1. **The central dogma of molecular biology.** k_{tr} – rate of transcription; k_{tl} – rate of translation; ΔT_{tr} – time delay for RNA production; ΔT_{tl} – time delay for protein production; δ_d – degradation rate.

Although most of these biobricks were created (table 6.1), testing and characterizing all the parts is still necessary. In particular the following experiments could be of interest in the future:

a) Regulation of transcription delay (ΔT_{tr}) using artificial introns

Others have shown that the size of the transcript may be used for increasing the transcriptional delay (69). However, this makes the transcript very large. An alternative approach may be the use of one or several artificial introns whereby the RNA would require a larger processing time before being exported from the nucleus.

Table 6.1 – **Biobricks for post-transcriptional regulation**.

Group	Name	Size	Stop	3n
Intron	Intron Start	9	No	Yes
	Intron Stop	40	No	No
Codon Usage	Good Codon Usage Linker	72	No	Yes
	Poor Codon Usage Linker	72	No	Yes
RNA Interference	miRNA scrambled	96	Yes	Yes
	RNAi against Good Codon Usage Linker	96	Yes	Yes
	miRNA against Good Codon Usage Linker	96	Yes	Yes
	RNAi against spacer1	96	Yes	Yes
	miRNA against spacer1	96	Yes	Yes
	Mirtron mir-62	64	Yes	No
	Target for mir-62	34	Yes	No
	Mirtron mir-1003	62	No	No
	Target for mir-1003	50	No	No
RNA Half-life	Au-Rich Element	21	No	Yes

b) Regulation of RNA degradation using Au-rich elements (AREs)

It is well established that au-rich elements contribute to the regulation of the mRNA half-life (70), however this tool has not yet been characterized systematically (i.e. the half-life coded using one or several copies of the ARE). We have experimentally confirmed that a lower EGFP fluorescence is obtained when the RNA contains an ARE in comparison with non-ARE containing RNAs, but a more systematic quantification is still required.

c) Regulation of translation delay (ΔT_{tl}) using several copies of a poor codon usage linker

The ribosome translation speed is codon dependent (71) and, as optimization of the codon sequence increases the protein levels obtained with each transcript, conversely, using a poor codon usage should delay the process of translation, which may be used in synthetic biology to manipulate that parameter.

Although time contraints prevented developing this project further, the new parts pave the way for the creation of a more specialized biobrick collection to be used in conjunction with the published library (see table 6.1 and sequences in **Annex 1**).

6.2 Article 2

Using an IRP activity dual-fluorescence reporter in a BNL CL.2 and RAW 264.7 surrogate model to hepatocytes and Kupffer cells co-culture

In this work we have made two main contributions to the field: 1) we establish a transiently transfectable iron sensor that we test in the context of liver cell iron metabolism; 2) we use BNL CL.2 and RAW 264.7 as a surrogate for hepatocyte-kupffer cell communication. Based on these, we propose the following lines of research:

a) Improving the eukaryotic IRP binding activity sensor

For the improvement of the eukaryotic iron sensor there are two main factors to consider: 1) the half-life of the proteins of the sensor and 2) the topology of the network by the implementing inhibitions of TI by IG and IG by TI as discussed in **Article 2**.

In our implementation, each protein of the IRP binding reporter has a long half-life (predicted to be over 72 hours). Shortening the half-life to a couple of hours by inserting a PEST Biobrick in the C-terminus of each protein would allow a time-course analysis of IRP binding activity.

Another improvement, as discussed in **Article 2**, would be the establishment of a topology such as presented in figure 4.2 A. The modeling performed in **Article 2** for topology 2 is valid for both transcriptional and post-transcriptional forms of inhibition. Although stronger inhibitions are likely to be obtained if transcriptional inhibition is used (and therefore probably a higher signal to noise ratio), I believe a low inhibition by a post-transcriptional mechanism such as inhibition of ribosome scanning by tethering of the proteins using Biobricks for MS2 and for λ N peptide may be more interesting since it should have a faster response time and, therefore, may be used in conjunction with the PEST sequences for a reporter with a time-course capability (see figure 4.2B).

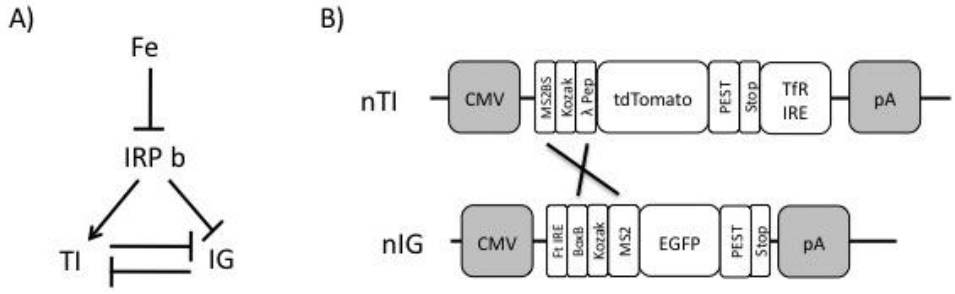

Figure 4.2. **Topology (A) and plasmids (B) schemas for improvement of an eukaryotic IRP binding activity sensor.**

b) Establishing a prokaryotic IRP binding activity sensor.

In addition to the eukaryotic IRP activity reporter, it would be interesting to have a prokaryotic IRP activity reporter. The implementation of the reporter (see figure 4.3A and 4.3B) varies slightly from that of the eukaryotic system because a clonal variant with IRP under a synthetic promoter may be easily obtained and the transcriptional inhibition (such as that of tetR) is faster than in eukaryotes. Such a reporter in a prokaryote may help in the study of the iron metabolism of e.g. phagosized Mycobacteriae (and therefore we prepare the prokaryote reporter with fluorescent proteins for which the absorption/emission spectra are sufficiently different from tdTomato and EGFP used for the eukaryotic reporter). However, a prokaryotic reporter in an organism such as *E. coli*, may also provide a system to be used as an iron sensor system to detect iron levels in a solution.

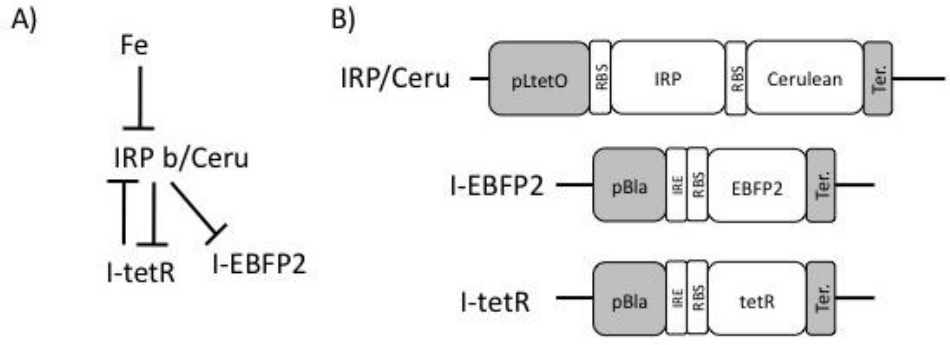

Figure 4.3. **Topology (A) and plasmids (B) schemas for a prokaryotic IRP binding activity sensor**.

c) Use of BNL CL.2 and RAW 264.7 to investigate the microarray observations

It will be particularly interesting to investigate which factors secreted by the RAW 264.7 macrophages affect the iron homeostasis of BNL CL.2 liver cells. To perform such analysis bioinformatics software such as Ingenuity systems pathway analysis tool may help to identify which pathways are being activated in BNL CL.2 and, therefore, identify which receptors-ligands may be implicated. In addition, it would be interesting to use gene over-expression or siRNA techniques to investigate which of the genes we found modulated may explain the modulation of the IRP binding activity reporter observed.

6.3 Concluding Remarks

For the present dissertation I have had the opportunity to collaborate with other 'wet' lab groups for the joint effort of establishing the Biobrick system at the Parc de Recerca Biomedica de Barcelona (PRBB) and with 'dry' lab groups, learning the intricacies of the dialog and jargon used in fields of science other than biology. In addition, I have been trained in cloning and DNA manipulation for the preparation of 'parts' from which the Biobrick library emerged as a tool that was useful not only for the performed work but provides a basis for future work. Using such 'parts' I implemented a 'device' (an IRP binding reporter) to use as a tool to study the biology of iron metabolism. The reporter was applied to the investigation on the modulation of the IRP binding in BNL CL.2 cells in response to co-culturing of RAW 264.7 macrophages. These experiments, along with the analysis of gene expression using microarrays allowed me to have experience on the use of high-throughput techniques to approach biological questions.

The established stand-alone Biobrick collection; the new model system; and microarray analysis pave the way for further research: an archetype of synthetic biology, whereby the new 'parts', 'devices', model 'systems' and high-throughput quantifications represent an accumulation of knowledge and tools to be used for further studies and engineering.

I believe this type of construction and characterization of devices and systems, along with the establishment of increasingly complete software and database systems will in the next couple of decades facilitate the modeling of both natural and synthetic systems allowing researchers to know how complex pathways behave without requiring the knowledge of all the participating components (i.e. allow the overlook of the synthetic and natural systems with a higher level of abstraction). This type of modeled data analysis should allow the engineering of potent biotechnological applications ranging from 'doctors in a cell' – i.e. programmed cell systems that correct pathogenic imbalances – to industrial bioproduction of drugs and biofuels, a set of achievements that will likely take place still in this century.

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7. ANNEX 1 – SEQUENCES OF SUPPLEMENTARY BIOBRICKS

>Intron start Biobrick RFC 23

aaggtaagt

>Intron stop Biobrick RFC 23

ctgattgacggtcacaaggctttcctctttttgcagggt

>Good codon usage linker Biobrick RFC 23

accggcggcagcgagcgccccctgaccggcggcagcgagcgccccctgaccggcggcagcgagcgccccctg

>Poor codon usage linker Biobrick RFC 23

acgggtggttcagaacgaccattaacgggtggttcagaacgaccattaacgggtggttcagaacgaccatta

>miRNA scrambled Biobrick RFC 23

ctgtaactcggaactggagaggccgctgctccccgcatctctgttgaactgggaacagagatgcggggagcagcggtttttcctgtctgacagcag

>RNAi against good codon usage linker Biobrick RFC 23

ctgtaactcggaactggagagggggcgctcgctgccgccggtttgaactgggaaaccggcggcagcgagcgccccttttttcctgtctgacagcag

>miRNA against good codon usage linker Biobrick RFC 23

ctgtaactcggaactggagagggggcgctttttgccgccggtttgaactgggaaaccggcggcaaaaagcgccccttttttcctgtctgacagcag

>RNAi against spacer 1 Biobrick RFC 23

ctgtaactcggaactggagaggggttcagttacttaacagaacttgaactgggaagttctgttaagtaactgaaccttttttcctgtctgacagcag

>miRNA against spacer 1 Biobrick RFC 23

ctgtaactcggaactggagaggggttcagtggtttaacagaacttgaactgggaagttctgttaaaccactgaaccttttttcctgtctgacagcag

>Mirtron mir-62 Biobrick RFC 23

aaggtgagttagatctcatatccttccgcaaaatggaaatgatatgtaatctagcttacagggt

>Target for mir-62 RFC 23

ggatccctgtaagctagattacatatcaggatcc

>Mirtron mir-1003 RFC 23

aaggtgggtatctggatgtggttggctctggcggtcctctcacatttacatattcacagggt

>Target for mir-1003 RFC 23

ttaattaaggatcctctcacatttacatattcacagggatccttaattaa

>Au-rich element RFC 23

atttatttatttatttattta