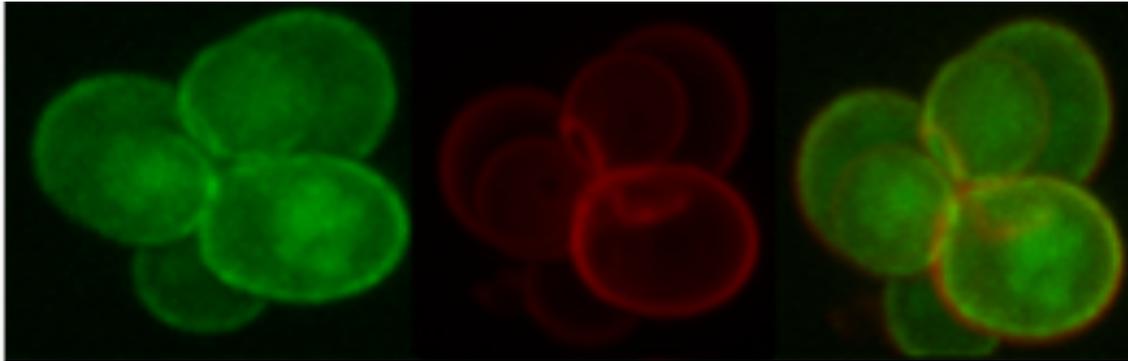




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Gpr1 mutagenesis libraries reveal novel mutations altering glucose signaling

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2017

MASTER'S THESIS 2017

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Master's Thesis 2017

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Cover: Confocal microscopy of Gpr1 in *Saccharomyces cerevisiae* with a GFP-tag in the C-terminus and the membrane stained with Concanavalin A.

Gothenburg, Sweden 2017

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Abstract

Glucose sensing in *Saccharomyces cerevisiae* is mediated by indirect or direct glucose sensing mechanisms. One direct mechanism is the GPCR system constituted by the Gpr1 receptor and its cognate G protein Gpa2. In response to glucose cAMP is generated which in turn binds to the regulatory subunit of PKA causing the catalytic subunits to dissociate and phosphorylate downstream targets. A parallel pathway constituted by Ras2 can also activate the same pathway. However, the downstream targets of both pathways are poorly characterized. This work aimed to generate a range of mutated Gpr1 receptors through development of a reporter assay utilizing the *SUC2* promoter which is repressed in high extracellular glucose concentrations and de-repressed when glucose becomes limiting, (below 5mM). The repression and de-repression mechanisms are regulated by factors binding the two activation sites of the *SUC2* promoter (termed SUC2A and SUC2B site). The activation sites were exploited and a set of *SUC2* promoters were engineered to drive the expression of a reporter construct in response to glucose. The same reporter constructs were used to screen a mutagenesis library for Gpr1 receptors with altered glucose activations profiles. In conclusion, we found that: 1) The *SUC2* pathway can be used as readout of the Gpr1 receptor. 2) Deletion of the SUC2A activation site provides an altered activation phenotype for P_{SUC2} and can be utilized for screening a Gpr1 mutagenesis library. 3) Gpr1 mutants with altered glucose activation profiles were generated with both higher basal activity and increased response to glucose. These receptors are hypothesized to be valuable tools in generating synthetic glucose bio-sensors.

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1 Introduction

Synthetic biology is driven by the potential of engineering new biological functions and has rapidly developed as a field during the last years. One of the most commonly used model organisms in synthetic biology is *Saccharomyces cerevisiae*. It is well studied and the complete genome is available [1]. *S. cerevisiae* is an eukaryotic organism with highly conserved cellular features and processes among most eukaryotes, and around 31 % of the genes have human homologs [1], and can thereby help understanding the biology of higher organisms. Genetic manipulations in *S. cerevisiae* are easy to perform since different toolkits are available [2]. Synthetic biologists have divided the complex parts of the cells into components with predictable interactions and thereby ease the prediction of function when assembling different parts [3].

Synthetic biology has a great potential in developing synthetic pathways in human cells to enable treatments for different diseases. Diabetes is a disease with over 400 million adults affected and the number is predicted to increase [4]. In type 1 diabetes the β -cells have lost the ability to produce insulin in response to glucose and in type 2 diabetes, glucose sensing and insulin release is impaired [5]. In an attempt to treat this disease using synthetic biology, Fussenegger and colleagues have developed a synthetic pathway, linking glucose sensing to release of glucose lowering peptides in human embryonic kidney 293 (HEK-293) cells [5]. The cells were microencapsulated in alginate beads and transplanted into mice with either type 1 or type 2 diabetes. The mice with type 1 diabetes were able to self-sufficiently abolish persistent hyperglycemia (high blood sugar) and the mice with type 2 diabetes had improved glucose-stimulated insulin release and glucose tolerance. This system has the potential to contribute to a treatment for diabetes. The downside of the system is that no direct glucose sensor for the physiological relevant concentrations was identified. Instead, the regulation of the system is controlled through indirect glucose sensing utilizing voltage-gated Ca^{2+} -channels and a calcium-responsive promoter [5].

1.1 Aim

The aim of this project was to generate a range of receptors with different sensitivity which potentially could be used as a direct glucose sensor to be integrated in a mammalian circuit producing insulin/glucagon in response to glucose. Specific aims with this project were to: 1) Develop a reporter assay to gauge Gpr1 activity. 2) Generate a Gpr1 mutagenesis library. 3) Combining aims 1 and 2 to isolate and characterize mutants with altered glucose signaling.

1.2 Limitations

The end goal was to integrate the receptors in a mammalian circuit for a glucose homeostasis network. However, it was considered a high risk of failure and instead the focus was shifted to the yeast receptor characterization. The integration of the Gpr1 mutants in mammalian cells were therefore beyond the scope of this report.

2 Background

2.1 Glucose metabolism

Glucose is the preferred energy source for *S. cerevisiae* and the glucose metabolism is dependent on several different pathways. The Snf1/Mig1 repression pathway is responsible for negative regulation of genes involved in glucose metabolism and utilizing alternative sugars [6]. The Rgt2/Snf3 induction pathway is responsible for the glucose uptake. The Rgt2 receptor is a sensor for high glucose concentrations, while the Snf3 receptor sense low glucose concentrations [7]. The third glucose pathway is the cAMP-PKA pathway which regulates cell growth, carbon storage, differentiation, etc and senses glucose both extracellularly and intracellularly [8] (Fig 1).

The cAMP-PKA pathway is initiated by glucose activation of adenylate cyclase (Cyr1) through the Gpr1/Gpa2 pathway or through the parallel Ras1/Ras2 pathway [9, 10]. Cyr1 converts ATP into cAMP, which binds the regulatory subunit of PKA, Bcy1. This leads to the release of

the catalytic subunits of PKA, Tpk1, Tpk2 and Tpk3, and thereby their activation [11]. PKA regulates several downstream targets involved in transcription, metabolism and cell cycle progression [12, 13]. The Gpr1/Gpa2 pathway consists of the G-protein coupled receptor (GPCR) Gpr1, which in response to glucose activates Gpa2 which encodes a downstream G-protein. The active Gpa2 further triggers the activation of Cyr1 [9].

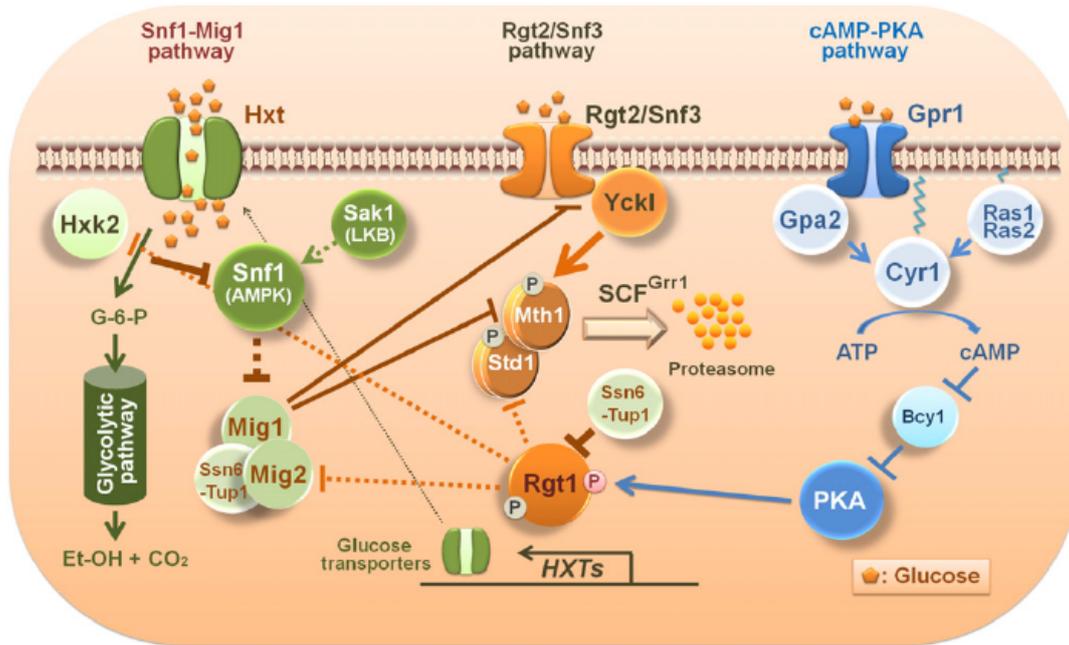


Figure 1: Three different signaling pathways for glucose in yeast and the internal crosstalk. In the presence of glucose the Rgt1 repressor is phosphorylated and thereby released from the upstream region of HXT genes resulting in expression [14]. The arrows indicate induction, while the flat lines indicate inhibition. The dotted line indicates a potential induction or inhibition. The different colors represent the different pathways. Image is adapted from Kim, 2013.

2.2 G-protein-coupled receptor 1 - Gpr1

The Gpr1 receptor is a member of the GPCR family and was discovered 1998 [15] and it has been shown to be activated by glucose and sucrose. However, the potency of sucrose is much higher compared to glucose, the reported EC_{50} for sucrose is 0.5 mM and for glucose 20 mM [16]. The Gpr1 receptor consists of 961 amino acids and has seven transmembrane domains (denoted TM I-VII), three extracellular loops (denoted EC I-III), three intracellular loops (denoted IC I-III) with a long third intracellular loop and long C-terminal tail (Fig 2) [17, 15].

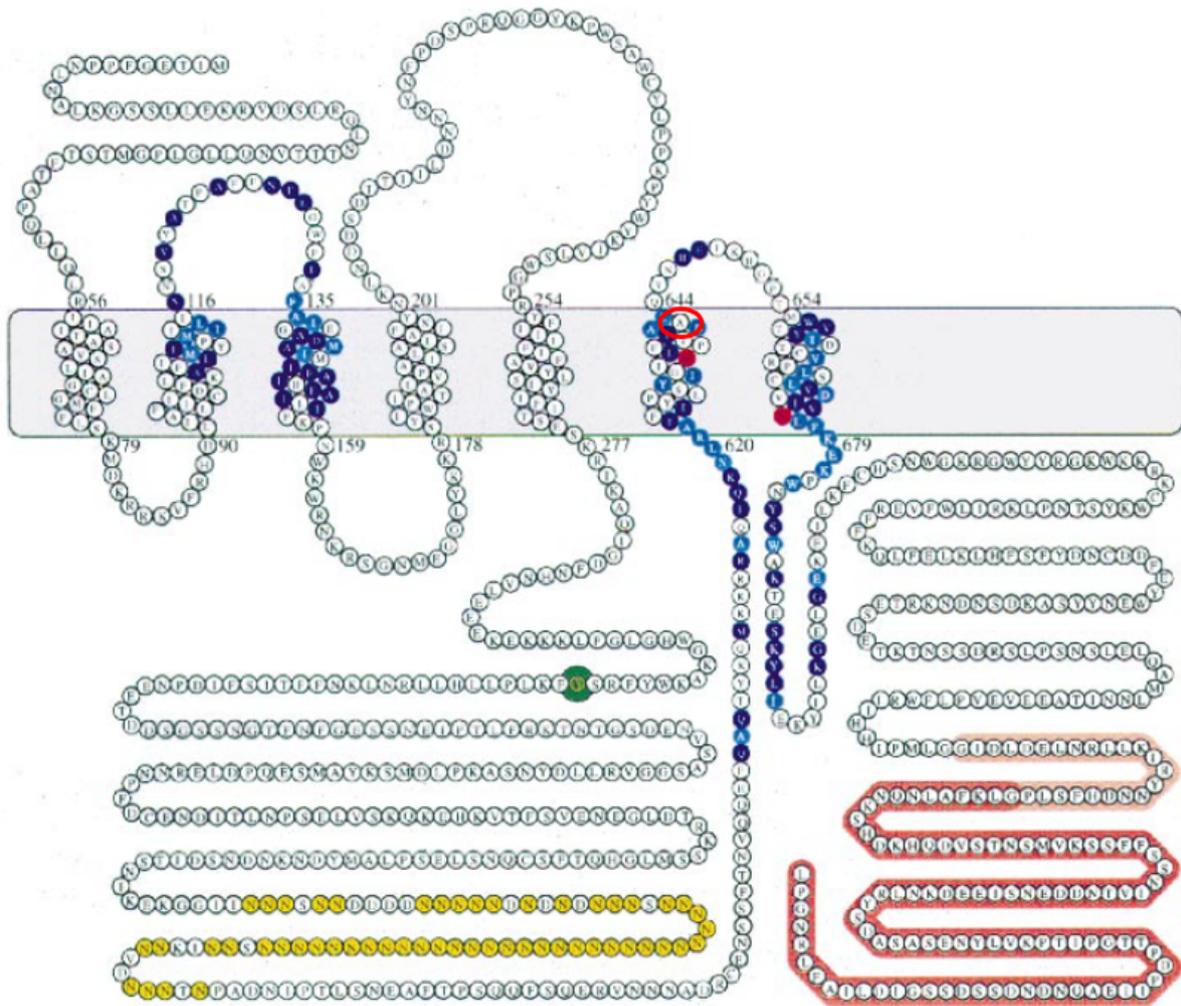


Figure 2: *The structure of the Gpr1 receptor. The amino acid 640, previously shown to be important for glucose interaction or induction [16], in TM VI is marked with a red circle, the other colors are not of importance. Image is adapted from Kraakman, 1999.*

The Gpr1 receptor has previously been mutated using a method where amino acids in TM VI were altered, since this region is considered to be important for ligand binding and it was shown that the potency seems to change by mutating amino acids in TM VI [16]. Moreover, the mutation Gpr1-A640C resulted in deficient cAMP signaling in response to glucose but not to sucrose, indicating the importance of the amino acid A640 for glucose interaction or induction of the cAMP-pathway in response to glucose [16]. Since Gpr1 binds glucose with a lower potency than sucrose it is interesting to evaluate if it is possible to engineer this receptor to increase the affinity to glucose and thereby make it suitable as a glucose sensor in a physiological relevant

setting. A potency of glucose between the physiological relevant range, 4 and 6 mM [18], would be of interest and could potentially be obtained by mutating Gpr1.

2.3 Glucose responsive promoters

There are several glucose responsive promoters in *S. cerevisiae* and one group of proteins induced by these promoters are the hexose transporters (HXTs). Some of the *HXT* promoters are repressed and some derepressed at high glucose concentrations [19]. The repression mechanism is mediated for instance by the Rgt1 repressor [20].

The *SUC2* promoter is also induced by glucose and has previously shown to be induced by Gpr1 signaling [21, 22]. The *SUC2* gene encodes for an invertase enzyme that hydrolyses sucrose into glucose and fructose [23]. The *SUC2* promoter (P_{SUC2}) is repressed at high glucose concentrations or in the absence of glucose and derepressed when the concentrations of glucose is below 5 mM, making it a favorable promoter when studying glucose metabolism during the indicated concentrations [19].

The P_{SUC2} has two activations sites, SUC2A and SUC2B, present 499 and 442 bp upstream of the *SUC2* gene, in the cis-regulatory element [24]. Several regulatory elements of P_{SUC2} have been characterized [24]. However, the interplay between these factors are not well understood. The zinc finger containing transcription factors Mig1 and Mig2 binds both the SUC2A and SUC2B sites during high glucose concentrations and thereby repress induction of *SUC2* [25]. When glucose becomes limiting, Mig1 and Mig2 are phosphorylated and *SUC2* expression is induced [24]. During high glucose concentrations Rgt1 binds the SUC2B site, at similar sequence as the Rgt1 binding sites in *HXT* promoters, and thereby repress *SUC2* induction [22, 26]. During low glucose concentrations Rgt1 is phosphorylated and released from the SUC2B site leading to relieved repression (Fig 3) [21].

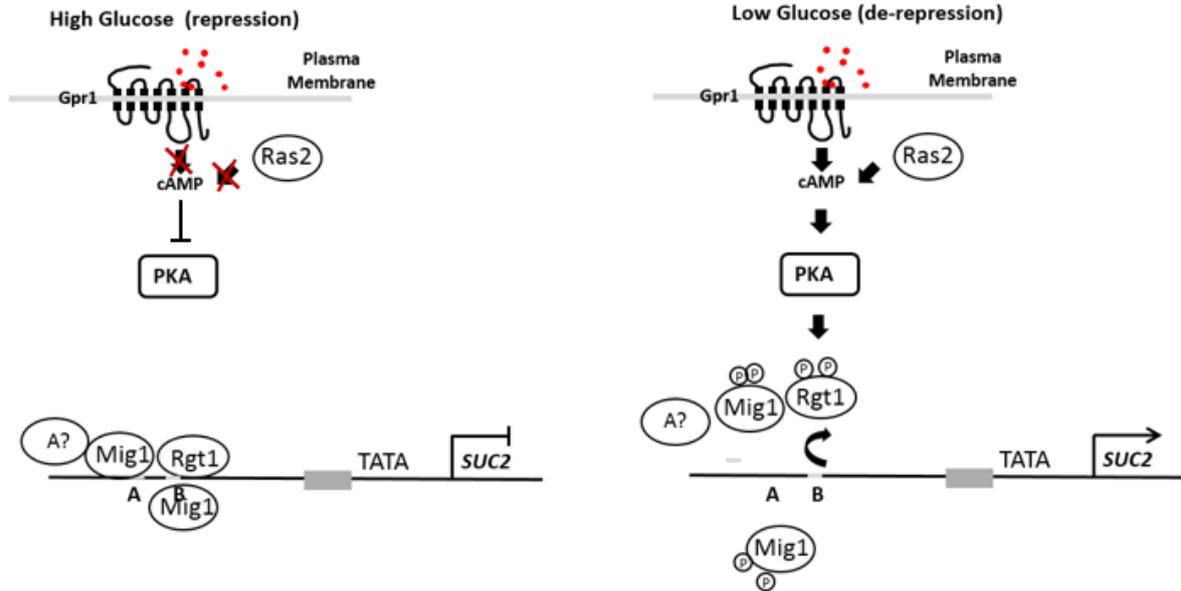


Figure 3: *Repression and de-repression of SUC2. High glucose concentration results in repression of SUC2, while low glucose concentration results in loss of repression of the two activation sites, SUC2A and SUC2B, and thereby induction of SUC2. A represent a potentially unknown factor involved in the repression of SUC2 [21].*

3 Methods

3.1 Plasmid construction

A yeast toolkit developed by Lee et al. 2015 (MoClo-YTK from Addgene) was used in this project and all constructs were assembled with golden gate assembly. The method is based on digestion and ligation, where each part (promoter, gene coding sequence, etc.) has a specific overhang complementary to the next part [3]. The constructs were designed in Benchling (www.benchling.com), which support golden gate assembly. The reaction mix for golden gate assembly was prepared as follows: 1 μ L of each DNA insert or plasmid, 2 μ L T4 DNA Ligase buffer (NEB), 1 μ L T7 DNA Ligase (NEB), 1 μ L restriction enzyme, either BsaI or BsmBI (10 000 U/mL from NEB), and water to bring the final volume to 20 μ L. The DNA inserts and plasmids were normalized to equimolar concentrations (50 fmol each). The reaction mixtures were incubated in a thermocycler (Table 1) [3].

Table 1: Program used for golden gate assembly.

Digestion	42°C	2 min
Ligation	16°C	5 min
Repeat	30 repetitions	
Digestion	55°C	7.5 min

The *BsaI* restriction enzyme is used to form cassette plasmids from part plasmids, typically for expression of a single gene. The *BsmBI* restriction enzyme is used to form multigene plasmids from cassette plasmids (Fig 4) [3].

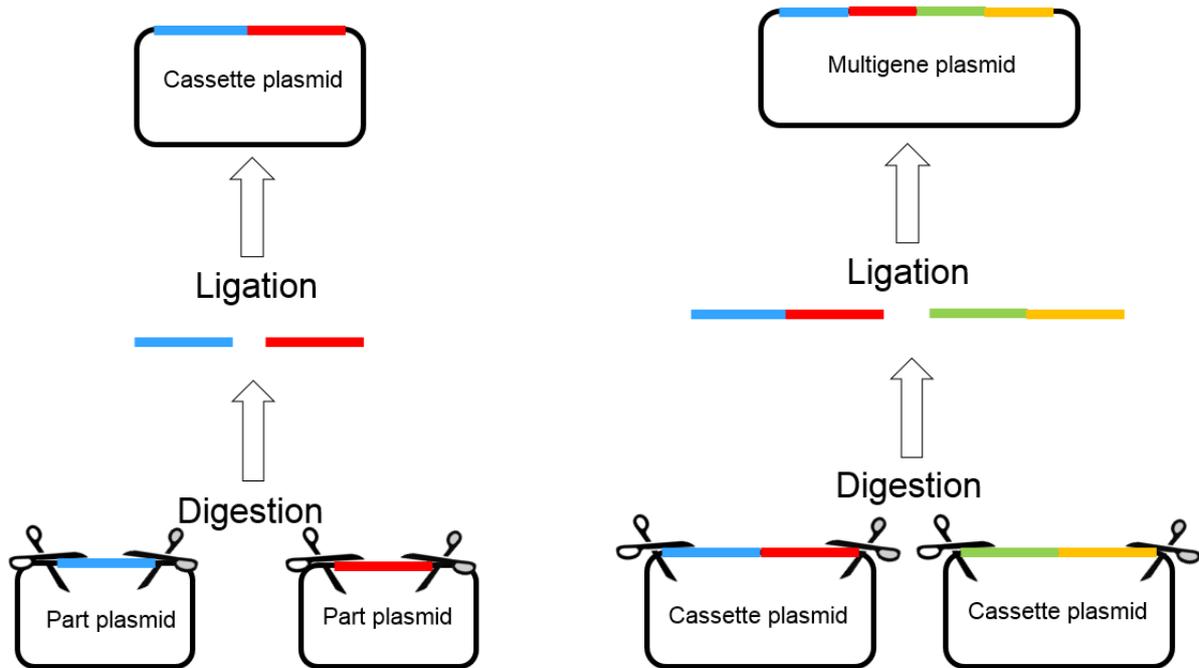


Figure 4: The principle of golden gate assembly. The part plasmid is digested using *BsaI* restriction enzyme and ligated into an acceptor vector to form a cassette plasmid containing all inserts (promoter, gene coding sequence, terminator, etc.) without additional nt. The cassette plasmids can further be digested with *BsmBI* restriction enzyme and ligated into an acceptor vector to form a multigene plasmid containing several gene coding sequence from the cassette plasmids without additional nt.

Golden gate assembly was used to create different reporter constructs (Appendix A.1), utilizing the *SUC2* promoter either with full length or with the two activation sites, *SUC2A* and *SUC2B*, deleted using a 12 nucleotides replacements according to Bu and Schmidt [1998]. It

was assembled with the reporter, *superfolder GFP* (*sfGFP*), the *SSA1* or *ENO1* terminator in the low-copy vector pWSP037. In addition the plasmids were further assembled with the *TEF1* promoter constitutively driving the expression of *mRuby* and with the *SSA1* terminator [3]. All further plasmids were also assembled using golden gate assembly (Appendix A.3).

3.2 Transformation

3.2.1 *E. coli*

DH5 α competent cells (Thermo Fisher Scientific) were used for all cloning experiments and the included protocol was followed [2]. However, for library generation One Shot[®] TOP10 Chemically Competent cells (Thermo Fisher Scientific) were used. Transformed cells were selected on Luria-Bertani (LB) with the appropriate antibiotics (ampicillin, chloramphenicol, or kanamycin). The plasmids were purified using new England BioLabs[®] Inc. “Monarch[®] Plasmid Miniprep Kit” [27]. Plasmids were verified using digestion with NotI (Thermo Fisher Scientific) and the fragments were separated by gel electrophoresis.

3.2.2 *S. cerevisiae*

Yeast colonies were grown overnight in YPD Broth (Teknova) with 2 % glucose or media supplemented with appropriated amino acid depending on the auxotrophic requirement. The cultures were diluted 1:6 with 2.5 mL of fresh media and grown for 4-6 hours. The cells were pelleted and washed once with water and twice with 1 M Lithium Acetate (Sigma). For transformation of plasmid, 0.5 μ g of DNA was used and 50 μ L of cells in 150 μ L of 50% PEG-4000 (Merck Schuchard) and 30 ng of salmon sperm DNA (Thermo Fisher Scientific). The transformation mixture was incubated at 42 °C for 30 minutes. Plasmids designed for chromosomal integration were digested with NotI (NEB) for 3 hours prior to transformation. In total 2 μ g of DNA was used for transformation and incubated at 30 °C for 45 minutes prior to incubation at 42 °C for 15 minutes. The mixture was pelleted and resuspended in YPD and incubated at 30 °C at 700 RPM for 2 hours. Finally the mixture was resuspended in water and plated on YPD plates or plates supplemented with appropriate amino acids [28]. For the Gpr1 library 15 μ g of DNA was used for the transformation.

3.3 Strains and growth media

The *S. cerevisiae* BY4741 strain was used for generating the different reporter strains and further for the generation of the mutagenesis libraries (Appendix A.4).

The BY4741 strain was grown in YPD broth or on YPD plates. The YPD plates were prepared as follows: 10 g of yeast extract (Sigma), 20 g of peptone (Sigma), 25 g of agar (Sigma) and water to bring the volume to 900 mL. The pH was adjusted to 5.2 with 33 % HCL prior to autoclavation. 100 mL of 20 % glucose was added to a final concentration of 2 % glucose and the YPD mixture were poured into plates (10-20 mL).

The reporter strains were grown on Yeast Nitrogen Base (YNB) plates supplemented with appropriate amino acids depending on the auxotrophic requirement. The plates were prepared as follows : 1X Yeast Synthetic Drop-out Medium Supplements (Sigma), 25 g of agar (Sigma) and water to bring the final volume to 800 mL prior to autoclaving. 100 mL of YNB (10X) and 100 mL of 20 % glucose (with the final concentration of 5 mM) were added and the mixture was poured into petri dishes (10-20 mL). The mutagenesis library strains were also grown on YNB plates but with 0.6 mM glucose.

The reporter strains and mutagenesis library strains were grown in YNB, 5 % glycerol (previously shown to repress P_{SUC2} [19]), 5 mM glucose and supplemented with appropriate amino acids overnight. The yeast cultures were pelleted and resuspended in media lacking glucose and grown for 4 hours prior to *SUC2* activation assay. The strains used for imaging were grown in YNB media with 2 % glucose supplemented with appropriate amino acids.

The YNB (10X) was prepared as follows: 50 g of ammonium sulphate (Sigma) and 17 g of yeast nitrogen base (Sigma) in 1 L of water. The solution was sterilized using vacuum filtration, with the pore size 0.45 μm (VWR). The 20 % glucose was prepared as follows: 200 g of D-(+)-Glucose (Sigma) was dissolved in 1 L of water and autoclaved.

3.4 *SUC2* activation assay

The *SUC2* activation assay was developed using the different reporter constructs and evaluating the response to different glucose concentration, depending on the activation of P_{SUC2} . The cells were pregrown according to section 3.3 prior to inoculation in different glucose concentrations. The excitation and emission wavelengths were measured on a Safire² (TECAN) for sfGFP at 485 nm/510 nm and for mRuby at 559 nm/600 nm. In addition OD_{600} was measured.

3.5 Gpr1 mutagenesis library generation and screening

The Gpr1 receptor was mutated using a kit: the JBS Error-Prone Kit. The included protocol was followed and 25 μ g of each primer in the primer pair EP-Gpr1 (Appendix A.5) and 30 ng of template was used [29]. The PCR product for the Gpr1 mutants were separated on a gel and the fragments with the right length were cut out and the DNA was extracted using new England BioLabs® Inc. “Monarch® DNA Gel Extraction Kit” [30]. The mutants were further assembled with the constitutive *SAC6* promoter [3], and the *ADH1* terminator in the high-copy vector pWSP038 (Appendix A.2). The mutagenesis library was pooled prior to transformation into yeast strains containing the reporter constructs.

The first selection step was based on growing the mutagenesis library on YNB plates supplemented with the appropriate amino acids with 0.6 mM glucose and utilizing the $P_{SUC2}LEU$ construct. The second selection step was based on the *SUC2* assay to screen the mutagenesis library and the plasmids for the mutants with altered phenotype in response to glucose were extracted using new England BioLabs® Inc. “Monarch® Plasmid Miniprep Kit” [27]. However, prior to addition of lysis buffer, cells were mechanically disrupted using 100 μ L glass beads and shaken at high speed for 30 seconds and resting on ice for 1 minutes, repeated 3 times. The supernatant was transferred to clean eppendorf tubes and the included protocol was followed to extract the plasmids.

Plasmids were sequenced using Sanger Sequencing by GATC and 25 pmol of each primer of Sequencing primer 1-16 (Appendix A.5) and 0.5 μ g of template DNA was used. The sequence

was aligned with wild type *GPR1*.

The Gpr1 mutants of interest were further assembled for chromosomal integration (Appendix A.2) and transformed into the URA-locus. In addition, the mutant A640C was included as a control.

3.6 PCR

The GFP-tag was amplified by PCR using Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB) with the primer pair GFP4a (Table 4) and 25 ng DNA template for 50 μ L reaction was used and the manufactures protocol was followed.

The mutant A640C was generated through PCR site-directed mutagenesis with the primer pair GPR1A640V (Appendix A.5) and the same protocol included in the JBS Error-Prone Kit was followed using 25 μ g of each primer and 30 ng of template DNA.

The verification of the chromosomal integration was performed following the same protocol for the GFP-tag amplification with the primer pairs URA3 5' and URA3 3'. However, the initial denaturation was changed to 2 minutes and 10 ng DNA template was used for a 20 μ L reaction.

3.7 Imaging

Gpr1 mutants of interest and the wild type Gpr1 were further assembled with a GFP-tag in the C-terminus on a low-copy vector (Appendix A.2) and transformed into yeast (Appendix A.4). Cell membranes were stained using Concanavalin A, Alexa Fluor® 647 Conjugate (Thermo Fisher Scientific) dissolved in NaH_2PO_3 buffer (Sigma). Cells were pelleted and resuspended with 0.1 mg/ml of Concanavalin A and incubated in room temperature for 30 minutes. The cells were washed with the NaH_2PO_3 buffer and resuspended in media. Cells were transferred to a 96-well plate and the images were acquired using a confocal Yokogawa (CV7000) with a

60x water immersion lens. For the visualization of GFP a 488 nm laser was used with maximum exposure for 400 ms. For the visualization of Concanavalin A a 650 nm laser was used with maximum exposure for 15 ms. The images were processed using ImageJ.

4 Results

4.1 *SUC2* activation assay development

The P_{SUC2} has been the subject of numerous studies and several regulatory factors have been identified [24]. Two such factors that seem to play a dominant role in *SUC2* regulation are the zinc finger containing transcription factors Mig1 and Mig2 which bind to a sequence of GC rich stretches termed the SUC2A and SUC2B site [25]. The Rgt1 transcription factor binds only to the SUC2B site and is required for full derepression of *SUC2* [26]. The derepression is mediated via phosphorylation of Rgt1 and subsequent disassociation from DNA in response to glucose [14]. Based on these findings a *SUC2* activation assay was developed to evaluate the reporter constructs to be used for monitoring Gpr1 activity in response to glucose. The strategy for the assay was based on glucose induction of the cAMP-PKA pathway by Gpr1 signaling and further induction of *SUC2* (Fig 5).

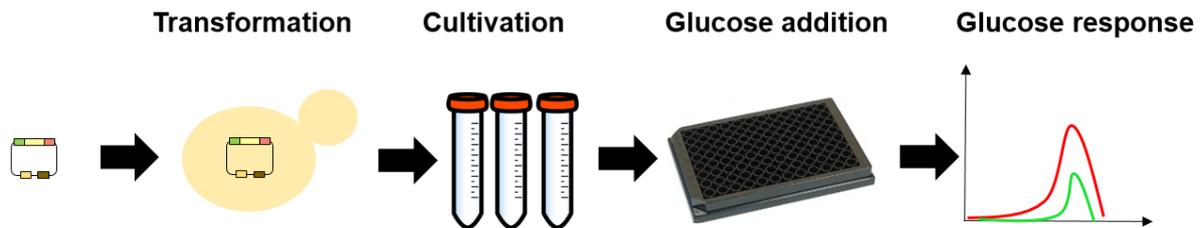


Figure 5: *The $SUC2$ activation assay. The reporter constructs were transformed into the yeast strains (Table 3). The cells were grown overnight in YNB media with minimal glucose and 5% glycerol and supplemented with the appropriate amino acids. The cells were pelleted and resuspended in media lacking glucose and grown for 4 hours. The cultures were transferred to a 96-well plate and different glucose concentration were added and incubated for 3-4 hours at RPM 850 and 30°C. The sfGFP signal was measured to evaluate the response to glucose and the mRuby signal or the OD_{600} was measured to enable normalization.*

4.1.1 Reporter construct- $P_{SUC2}sfGFP$

The first reporter construct evaluated consisted of the $P_{SUC2}sfGFP$ reporter, where the response to glucose generates a sfGFP signal through activation of P_{SUC2} (Fig 6).

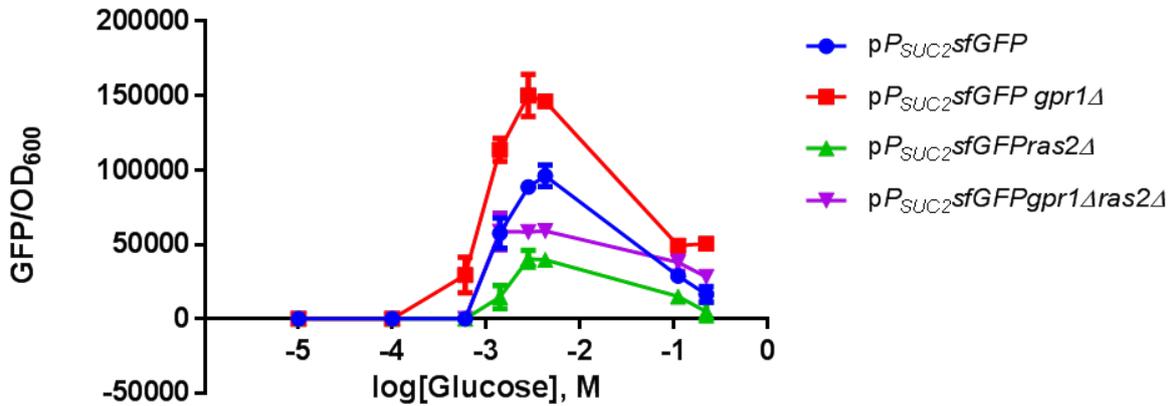


Figure 6: The dose-response to glucose for the reporter construct with the $P_{SUC2}sfGFP$. sfGFP expression and population density (OD_{600nm}) were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The sfGFP expression was normalized to the population density, represented on the y-axis. All data points and error bars represent the mean and standard deviation of duplicates and is representative for three independent experiments.

The $SUC2$ activation assay resulted in activation by glucose for the reporter construct, $P_{SUC2}sfGFP$ for all strain tested (Fig 6), and was therefore used for evaluating the rest of the reporter constructs. Each set of strains included a wild type reference strain, a $gpr1\Delta$ mutant, a $ras2\Delta$ mutant and $gpr1\Delta ras2\Delta$ mutant since the activation of the P_{SUC2} is dependent on both Gpr1 and Ras2 (Fig 3) [9, 10]. The phenotype for glucose activation of P_{SUC2} was not as distinct in the $ras2\Delta$ and the $gpr1\Delta ras2\Delta$ mutants as in the wild type and $gpr1\Delta$ mutants (Fig 6). There is a potential problem to normalize to the population density since there is a possibility that not all cells have taken up the plasmid. Hence, we sought to refine our reporter strain with an internal control.

4.1.2 Reporter construct - $P_{SUC2}sfGFP P_{TEF1}mRuby$

Using a $P_{SUC2}sfGFP$ reporter as a base, an additional constitutively expressed reporter, mRuby2 (a red fluorescent protein), was integrated on the same plasmid as an internal control (Fig 7).

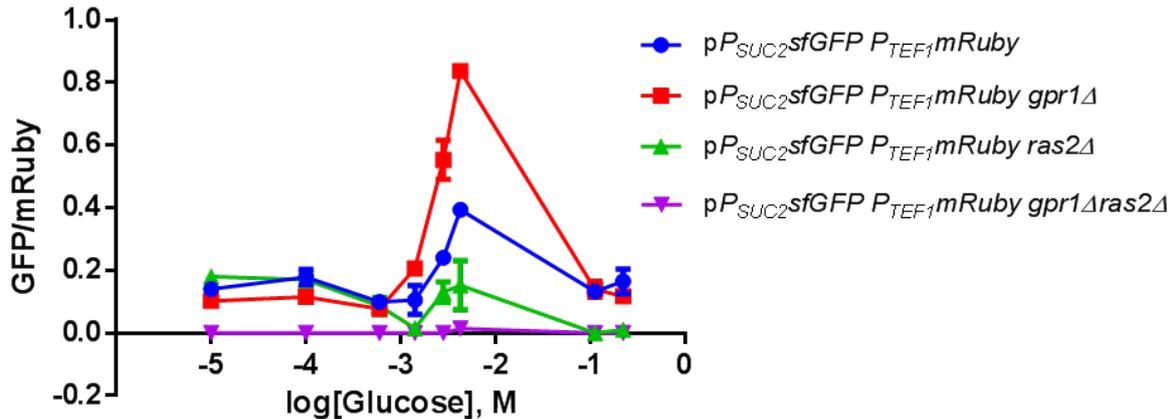


Figure 7: The dose-response to glucose for the reporter construct with the $P_{SUC2}sfGFP P_{TEF1}mRuby$. $sfGFP$ expression and $mRuby$ expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The $sfGFP$ expression was normalized to the $mRuby$ expression, represented on the y-axis. All data points and error bars represent the mean and standard deviation of duplicates and is representative for three independent experiments.

Similar to the construct with only $sfGFP$, this reporter was also activated by glucose in a Gpr1 and Ras2 dependent manner. The $gpr1Δras2Δ$ mutant showed no reporter activation. The $gpr1Δ$ mutant resulted in higher response to glucose compared to the wild type strain, while a very low response was observed for the $ras2Δ$ mutant (Fig 7). We conclude that the reporter with both $P_{SUC2}sfGFP$ and the internal control with constitutively expressed $mRuby$ generated a more accurate result for glucose activation. In all further experiments using reporter constructs, the $mRuby$ was employed for normalization.

4.1.3 $SUC2$ promoter mutants

In an attempt to identify downstream Gpr1 specific factors both the $SUC2A$ and $SUC2B$ activation sites were mutated. Hence, the two activation sites were investigated to further understand

regulation of P_{SUC2} and potentially increase the resolution between a wild type and a $gpr1\Delta$ mutant. Four different reporter constructs with $P_{SUC2}sfGFP P_{TEF1}mRuby$ with either wild type P_{SUC2} or the activations sites mutated were evaluated (Fig 8).

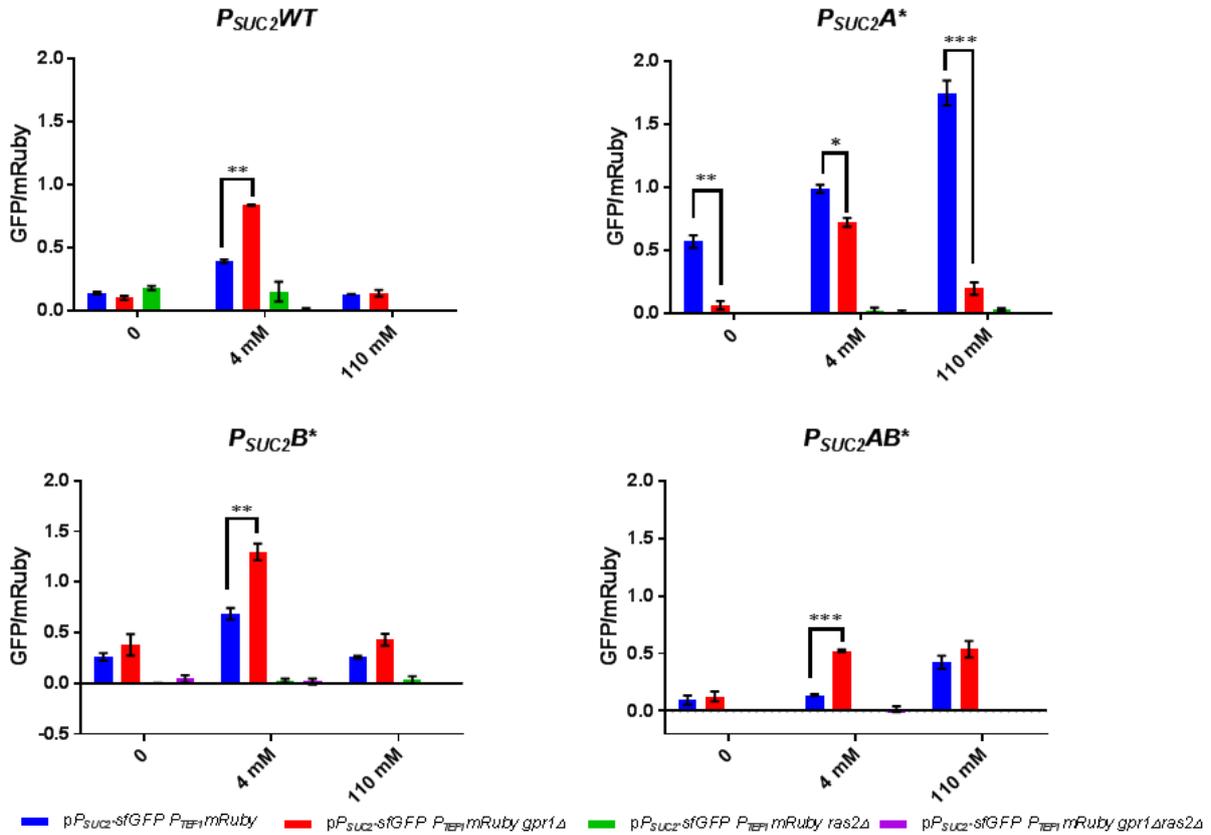


Figure 8: The repression and de-repression of the wild type P_{SUC2} , the $SUC2A$ activation site deleted, the $SUC2B$ activation site deleted and both the $SUC2A$ and $SUC2B$ activation sites deleted. $sfGFP$ expression and $mRuby$ expression were measured 3-4 hours after glucose addition represented on the x-axis. The $sfGFP$ expression was normalized to the $mRuby$ expression, represented on the y-axis. All data points and error bars represent the mean and standard deviation of triplicates and is representative for two independent experiments. The data was analyzed by two-tailed t-test, $*P < 0.006$, $**P < 0.002$ and $***P < 0.00002$

The wild type P_{SUC2} was repressed in the absence of glucose in all strains, de-repressed at 4 mM of glucose in the wild type and the $gpr1\Delta$ mutants, and repressed again at 110 mM of glucose in all strains. However the $ras2\Delta$ and $gpr1\Delta ras2\Delta$ mutants had very poor expression of the reporter (Fig 8). This phenotype differs from wild type P_{SUC2} when the $SUC2A$ activation site was deleted. The wild type strain had a higher basal activity in the absence of glucose and was not repressed at 110 mM of glucose while the other strains had similar phenotype to the wild

type P_{SUC2} . When the SUC2B site was deleted, the phenotype was similar to the wild type P_{SUC2} . However, when both the SUC2A and SUC2B sites were mutated there was a lack of distinct induction. To further investigate the importance of the activation sites dose-responses using 12 different glucose concentrations were performed.

***SUC2AB* promoter mutant**

The SUC2A and SUC2B activation sites in the P_{SUC2} were deleted (see Fig 3) and assembled with the $P_{SUC2}sfGFP P_{TEF1}mRuby$ construct and dose-response experiments were performed to increase the understanding of the regulation.

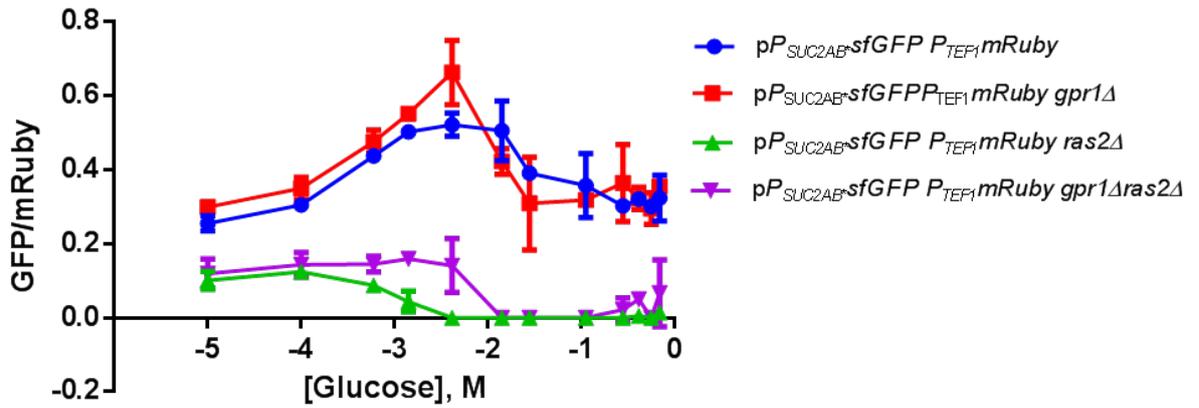


Figure 9: *Glucose dose-response using the $P_{SUC2AB}sfGFP P_{TEF1}mRuby$ reporter construct. $sfGFP$ expression and $mRuby$ expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The $sfGFP$ expression was normalized to the $mRuby$ expression, represented on the y-axis. All data points and error bars represent the mean and standard deviation of duplicates and is representative for three independent experiments.*

The activation of P_{SUC2AB} (where both activation sites were deleted) was similar for the wild type and the $gpr1\Delta$ mutants. Furthermore, the $ras2\Delta$ and the $gpr1\Delta ras2\Delta$ mutants failed to activate P_{SUC2AB} (Fig 9). To further investigate regulation of P_{SUC2} , the individual activation sites were deleted.

SUC2B promoter mutant

To investigate the role of the *SUC2B* activation site (see Fig 3), this site was deleted and dose-response experiments were performed.

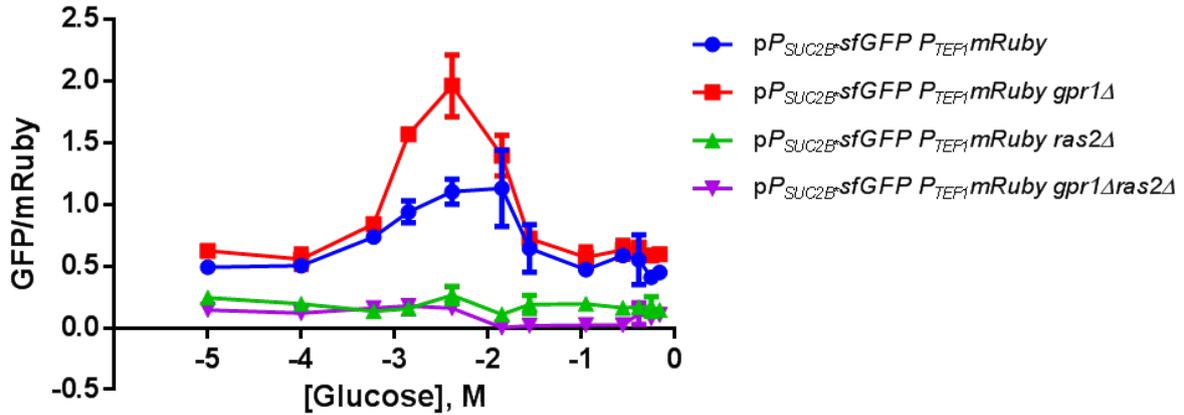


Figure 10: Glucose dose-response using the $P_{SUC2B}sfGFP P_{TEF1}mRuby$ reporter construct. *sfGFP* expression and *mRuby* expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The *sfGFP* expression was normalized to the *mRuby* expression, represented on the y-axis. All data points and error bars represent the mean and standard deviation of duplicates and is representative for three independent experiments.

Deletion of P_{SUC2B} resulted in a similar phenotype as the wild type P_{SUC2} (Fig 7). Thus, the *gpr1Δ* mutant gave a higher response to glucose compared to the wild type strain, while the response was abolished for both the *ras2Δ* and the *gpr1Δras2Δ* mutants (Fig 10).

SUC2A promoter mutant

To investigate the role of the *SUC2A* activation site (see Fig 3), this site was deleted and dose-response experiments were performed.

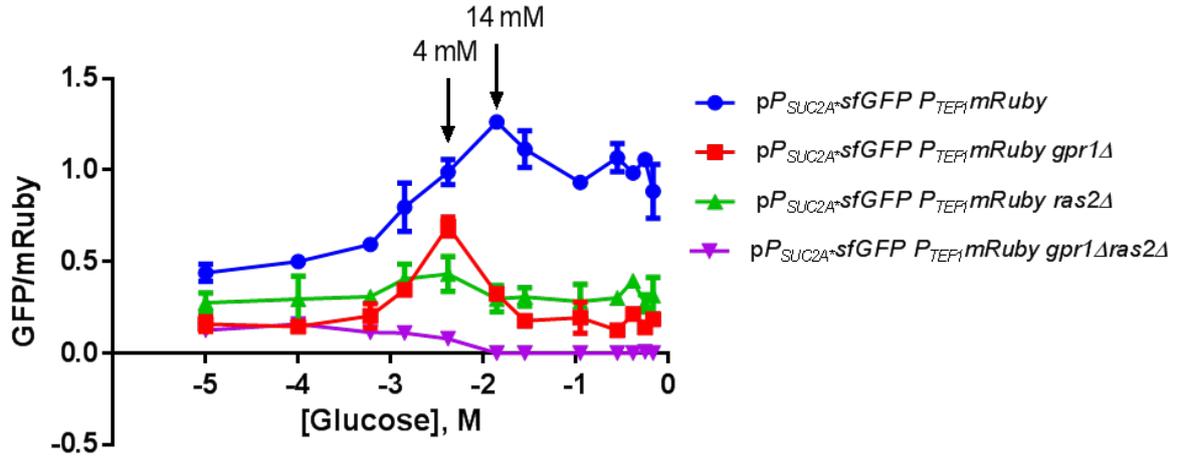


Figure 11: Glucose dose-response using the $P_{SUC2A}sfGFP P_{TEF1}mRuby$ reporter construct. *sfGFP* expression and *mRuby* expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The *sfGFP* expression was normalized to the *mRuby* expression, represented on the y-axis. All data points and error bars represent the mean and standard deviation of duplicates and is representative for three independent experiments.

P_{SUC2A} was activated by glucose in both the wild type strain and the *gpr1Δ* mutant. However, the wild type strain reached its maximum response at 14 mM and was not repressed at higher glucose concentrations. Interestingly, glucose caused very poor induction of the P_{SUC2A} reporter in the *gpr1Δ* strain. Neither the *ras2Δ* mutant nor the *gpr1Δras2Δ* mutant showed activation of P_{SUC2A} (Fig 11). The reporter construct with the SUC2A site deleted for the *gpr1Δras2Δ* mutant provided an increased assay window compared to the wild type strain. Most importantly, in this background, activation of the P_{SUC2A} reporter was associated with Gpr1 activity in response to glucose. Therefore, the *gpr1Δras2Δ* mutant with the P_{SUC2A} reporter was proceeded with for screening the Gpr1 mutagenesis library.

4.2 Library generation and screening

The reporter strain (*gpr1Δras2Δ P_{SUC2A}LEU pP_{SUC2A}sfGFP P_{TEF1}mRuby2*) was used to screen the Gpr1 mutagenesis library. The screening workflow was designed using both reporter systems (Fig 12).

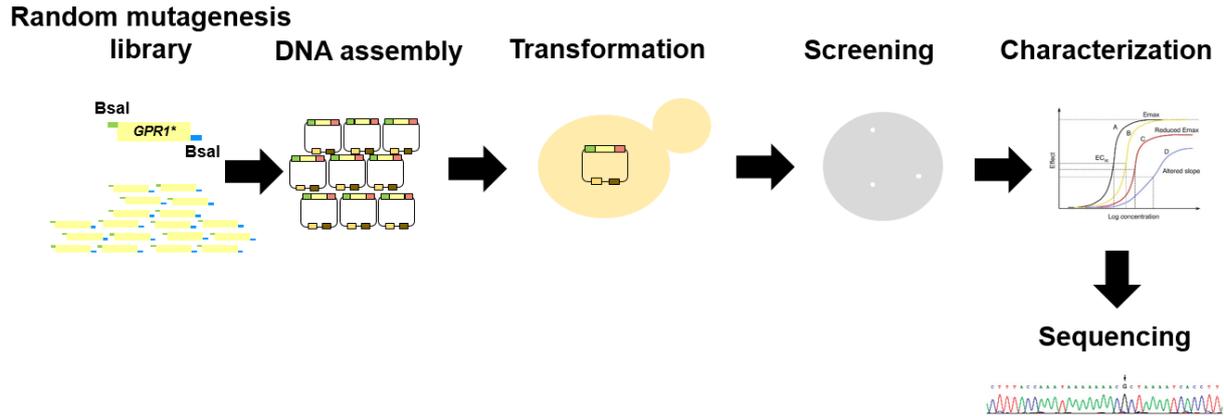


Figure 12: *The generation and screening strategy of the Gpr1 library. Gpr1 was mutated using random mutagenesis and assembled into a vector (Fig 19). Subsequently, it was transformed into the yeast strain with the reporter construct. Transformants were spread on plates lacking leucine (selection for yeast strain), histidine (selection for reporter construct) and uracil (selection for plasmid with mutated Gpr1). Cells were grown on low glucose concentration to select for mutants with increased potency of glucose. Resulting colonies with mutant Gpr1 were characterized by dose-response to glucose and candidates of interest were sequenced.*

The selection on plates with low glucose concentration resulted in 1100 colonies, where 400 were screened in response to glucose (Fig 12). Mutants with increased glucose response compared to wild type Gpr1 for 3 different glucose concentrations were further screened (Fig 12). The generation and screening of the Gpr1 mutagenesis library resulted in 8 candidates with altered glucose activation phenotype (Fig 13).

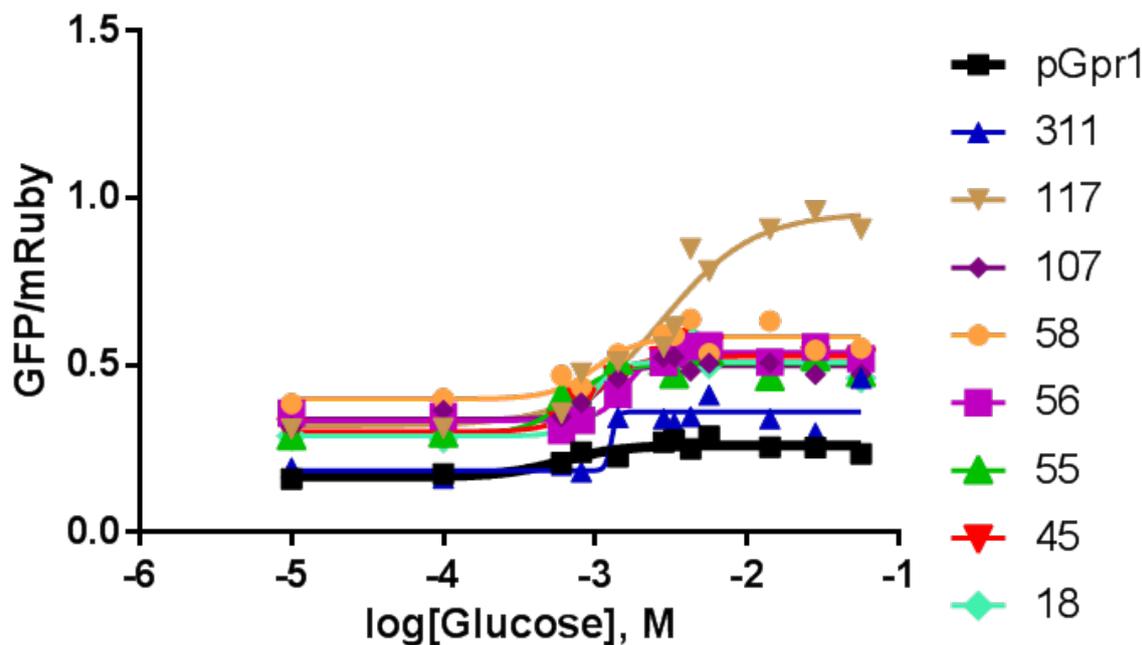


Figure 13: *The dose-response for the top 8 Gpr1 mutants. sfGFP expression and mRuby expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The sfGFP expression was normalized to the mRuby expression, represented on the y-axis. All data points are represented in duplicates and is representative for three independent experiments.*

All mutants except mutant number 311 had higher basal activity compared to wild type Gpr1. Moreover, the mutants also had increased maximum glucose response and especially mutant number 117 had a remarkable maximum response to glucose (Fig 13). Furthermore, the reporter construct $P_{SUC2A}sfGFP P_{TEF1}mRuby2$ showed a potency (EC_{50}) of 0.6 mM for glucose for wild type Gpr1. Glucose potency on mutant variant of Gpr1 was in a similar range. Plasmids from all 8 mutants were extracted and verified for the Gpr1 insert. The mutants containing Gpr1 in the plasmid were sent for sequencing which resulted in two different mutants, Gpr1-A640V (mutant number 117) and Gpr1-L238N (mutant number 56). They were further characterized using dose-response curves for 12 glucose concentrations (Fig 14).

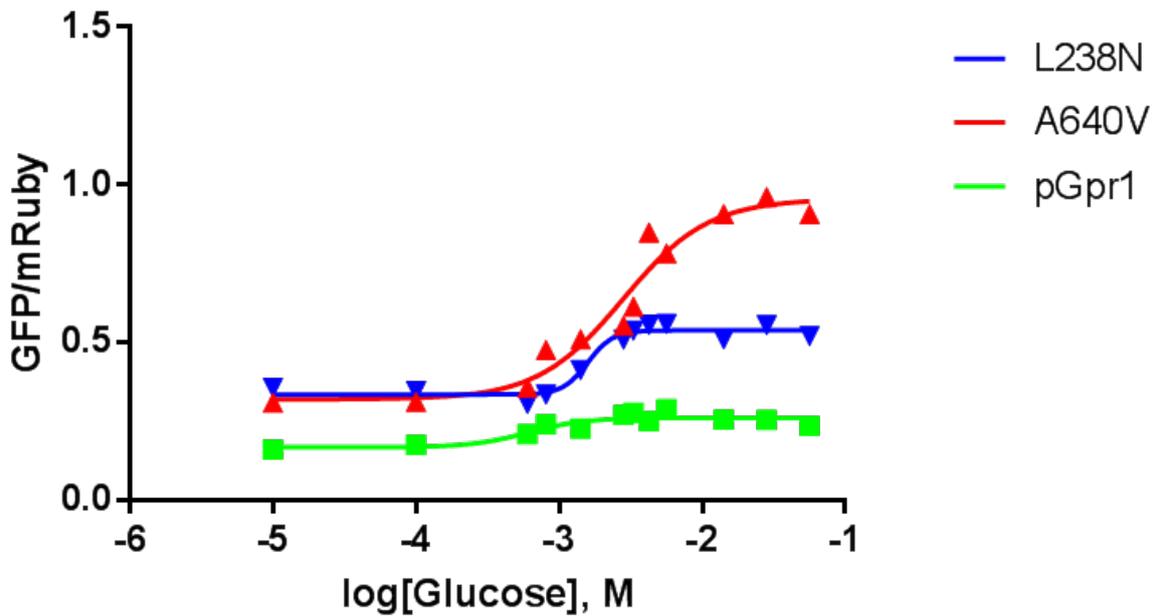


Figure 14: Glucose dose-responses for *Gpr1* mutants *L238N* and *A640V*. *sfGFP* expression and *mRuby* expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The *sfGFP* expression was normalized to the *mRuby* expression, represented on the y-axis. All data points are represented in duplicates and is representative for three independent experiments.

The dose-response curves for the two mutants showed a higher basal activity compared to wild type *Gpr1* and an increased maximum response to glucose, with *Gpr1-A640V* having the highest maximum response (Fig 14). The *Gpr1-L238N* is present in the second extracellular loop and the *Gpr1-A640V* in the sixth transmembrane domain of *Gpr1*. The mutants were further characterized by chromosomal integration in the *URA*-locus, to control for copy number since copy number may influence expression levels and thereby pharmacological response. The *Gpr1-A640C*, previously shown to have deficient cAMP signaling [16], was used as a negative control.

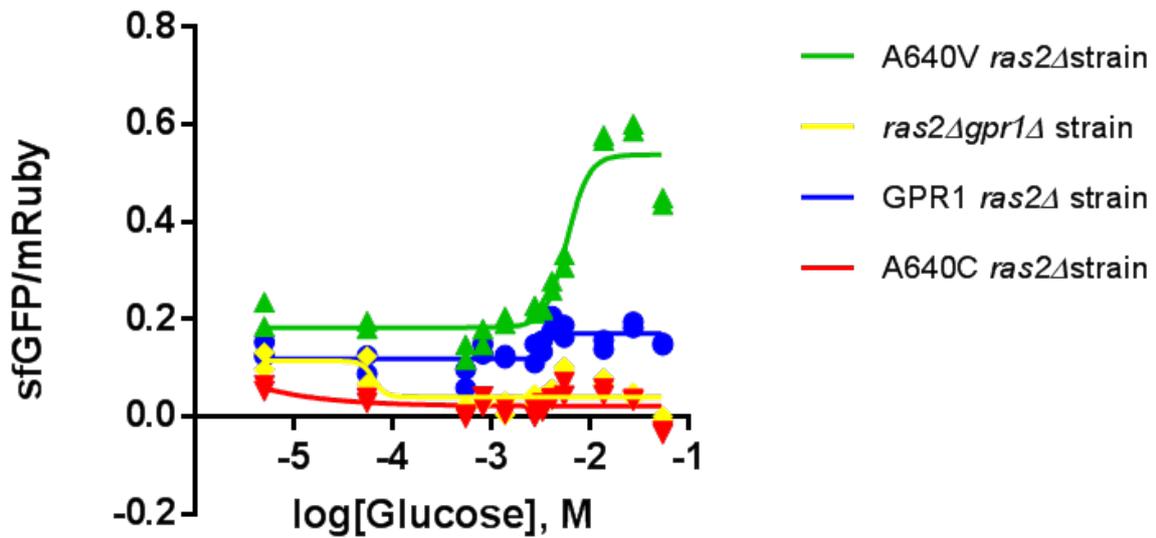


Figure 15: *Glucose dose-responses after chromosomal integration of wild type and mutant Gpr1 receptors. sfGFP expression and mRuby expression were measured 3-4 hours after glucose addition, with different concentrations of glucose, represented as the logarithm of the molar concentration on the x-axis. The sfGFP expression was normalized to the mRuby expression, represented on the y-axis. All data points are represented in duplicates and is representative for three independent experiments.*

Wild type Gpr1, Gpr1-A640V and Gpr1-A640C were integrated in the URA-locus and dose-responses to glucose were evaluated (Fig 15). Gpr1-A640V and wild type Gpr1 had similar glucose activation phenotypes as observed in Fig 14, ruling out that differences in copy number caused the phenotype. Neither Gpr1-A640C nor the *gpr1Δras2Δ* mutant were activated by glucose. To further characterize the mutants they were GFP-tagged to investigate subcellular localization.

4.3 Imaging

The wild type Gpr1, Gpr1-A640V, Gpr1-A640C and Gpr1-L238N were assembled with a GFP-tag in the C-terminus to enable localization of the receptors. The GFP-tagged plasmids were transformed into *gpr1Δ* and *gpr1Δras2Δ* mutants and analyzed using confocal microscopy (Fig 16).

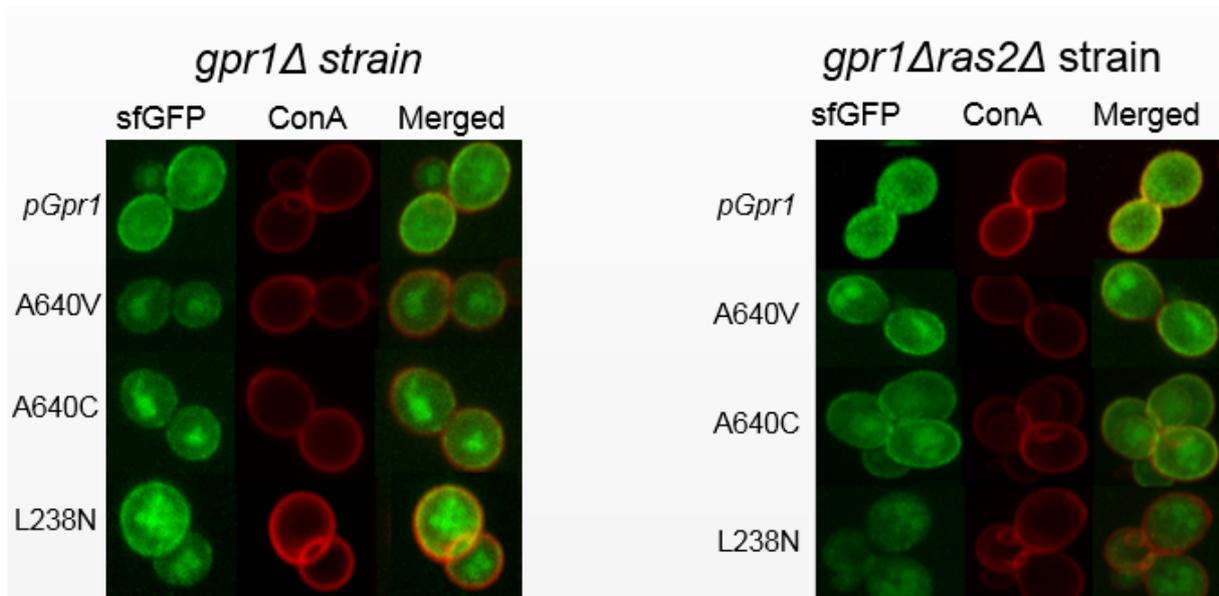


Figure 16: Subcellular localization of *Gpr1*-sfGFP. The *Gpr1* receptors were GFP-tagged and membranes were stained with concanavalin A (Con A) Alexa Fluor 647 and the images were merged.

The *Gpr1* receptors, both wild type and the mutants showed membrane localization in both the *gpr1Δ* mutant and the *gpr1Δras2Δ* mutant.

5 Discussion

5.1 *SUC2* activation assay

We hypothesized that glucose activation of P_{SUC2} using the reporter construct with P_{SUC2} sfGFP, would result in glucose activation in the wild type, *gpr1Δ* and *ras2Δ* mutants, since the reporter activation is dependent on both *Gpr1* and *Ras2* [9, 10]. This is supported by our data (Fig 6). Therefore, the *gpr1Δras2Δ* mutant was hypothesized to have no activation in response to glucose. The reporter construct P_{SUC2} sfGFP showed induction in all strains tested. However, it is not ideal to normalize the sfGFP expression to population density and there might be a problem that not all cells have taken up the plasmid or lost it during replication. In addition, the cell size might distort the readout and the *gpr1Δ* mutant has earlier shown to alter cell morphology and the cells are smaller compared to the wild type strain [31]. The problem was resolved by refining the reporter constructs with an internal control. The constitutive mRuby2 was used as

the internal control in the reporter construct with $P_{SUC2}sfGFP$ and thereby sfGFP expression was only normalized to cells containing the reporter. Since glucose activation was abolished in the $gpr1\Delta ras2\Delta$ mutant this supports our original hypothesis for the importance of these two proteins in activation of the P_{SUC2} reporter with the internal control (Fig 7).

The response to glucose was higher in the $gpr1\Delta$ mutant compared to the wild type strain. This is in line with previous studies showing that Ras2 is upregulated in a $gpr1\Delta$ mutant [32]. Moreover, the low activation in the $ras2\Delta$ mutant indicates the importance of Ras2 for induction of $SUC2$.

The activation sites of P_{SUC2} were deleted to investigate the regulation of the promoter. When both the activation sites were deleted, the activation phenotypes were similar for the wild type and the $gpr1\Delta$ mutants (Fig 9). It could be due to relieved repression from the SUC2B site or that a $gpr1\Delta$ mutant fails to activate the mutated SUC2A site (Fig 3). However, this needs to be further investigated. When the SUC2B activation site was deleted the $gpr1\Delta$ mutant had higher response to glucose compared to the wild type strain. A possible explanation is that Gpr1 is required to relieve repression from the SUC2B site, potentially through phosphorylation of Rgt1 (Fig 3) [21]. The activation of P_{SUC2A} , with the SUC2A site deleted, resulted in change of activation phenotype. The wild type strain reached maximum at 14 mM glucose and was not repressed by addition of more glucose (Fig 11). This result indicates that deleting the SUC2A activation site results in expression of invertase at high glucose concentrations (Fig 3). Hence, the SUC2A site is more important for repression of $SUC2$ than previously thought. The unaltered phenotype when deleting the SUC2B site compared to the wild type P_{SUC2} indicates that this site is less important for $SUC2$ regulation.

The reporter strain ($P_{SUC2}LEU$ $pP_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$ $gpr1\Delta ras2\Delta$) was used for screening the Gpr1 library since there is a clear assay window in this background (Fig 11). Hence, any change in phenotype for the activation of P_{SUC2A} is most likely linked to the Gpr1 mutations.

5.2 Gpr1 mutations

The Gpr1 mutagenesis library resulted in 8 different mutants with altered glucose activation profiles compared to the wild type Gpr1 (Fig 13). However, out of these 8 mutants only 3 contained the Gpr1 insert in the plasmid while the other 5 had "empty" plasmids. Hence, changes in glucose activation profile was not due to a mutation in Gpr1 for these 5 mutants. It could be due to suppressors which can enable other pathways to be activated, allowing growth on low glucose concentrations and induction of *SUC2* [33]. Mutants without Gpr1 inserts had similar glucose activation profiles with a higher basal activity but no distinct increased response to glucose suggestion they are constitutively expressing *sfGFP* potentially through another pathway. The three colonies containing a plasmid with Gpr1 insert resulted in the two different mutations A640V (mutant 117) and L238N (mutant 56): Moreover, the same mutations were obtained from a second mutagenesis library with site-specific mutations synthesized by Twist Bioscience. However mutant 55 contained wild type Gpr1. Both Gpr1-A640V and Gpr1-A640C had a higher basal activity compared to wild type Gpr1 (Fig 14) indicating, that both mutations result in constitutively active Gpr1. The selection of mutants by growing them on low glucose concentrations favor mutants with either increased potency for glucose or higher basal activity. Interestingly, Gpr1-A640V had a high maximum response to glucose. The Gpr1-A640C has previously been shown to have deficient response to glucose [16], indicating the importance of the amino acid in position 640 in Gpr1 for glucose interaction or signaling. Hence, Gpr1-A640C was used as a control when integrating the mutants in the URA-locus. Gpr1-L238N was regarded less interesting and not integrated in the genome since its glucose activation profile indicates that it was constitutively active and not activated in response to glucose.

The Gpr1 mutagenesis library was assembled on a high-copy vector since expression levels were titrated to match expression of endogenous wild type Gpr1, and the Gpr1 on a low-copy number vector resulted in insufficient expression (David Öling, personal communication). To control that the result was not a side effect of the copy number the mutants were integrated in the URA-locus. Dose-response curves were generated and Gpr1-A640V resulted in a similar glucose activation phenotype as observed when overexpressed (Fig 15). This indicates that the high response to glucose for Gpr1-A640V was not a side effect of copy number. Moreover, Gpr1-A640C showed no response to glucose as earlier shown [16].

All Gpr1 receptors, both the mutants (Gpr1-A640V, Gpr1-A640C and Gpr1-L238N) and the wild type Gpr1 were localized to the membrane (Fig 16). The difference of localization of the GFP expression intracellularly in the mutants compared to the more evenly expressed GFP in the wild type Gpr1 could be due to the higher basal activity in the mutants. Thereby they have a higher turnover and the Gpr1 receptors are degraded and transported to the vacuoles. Moreover, Gpr1-A640V had similar membrane localization compared to the other mutants, indicating that the higher dose-response to glucose observed in Fig 14 is not due to overexpression of the Gpr1 receptor but linked to the mutation. The localization of Gpr1-A640C to the membrane further supports role of amino acid A640 for glucose interaction or signaling [16].

The potency for glucose using the *SUC2* activation assay with the reporter construct $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$ was 0.6 mM for both wild type Gpr1 and for the mutants. It has previously been shown that the potency of glucose on Gpr1 is 20 mM. However this was shown in another assay (cAMP-assay) [16]. The result from the *SUC2* activation assay indicates that the Gpr1 already has a physically relevant potency for glucose. However, there is no information of this potency or maximum response in a potential mammalian circuit. Therefore, the next step is to further investigate different mutants of the amino acids in position 640 to generate a set of receptors with different sensitivity to glucose and further to integrate in a mammalian circuit to tune the regulation of the circuit.

6 Conclusion

The *SUC2* pathway can be used as a readout of Gpr1 activity using a *sfGFP* reporter gene. However, an internal control in the reporter plasmid was important for a robust readout. Moreover, deletion of the *SUC2A* activation site provided an altered activation phenotype of P_{SUC2} which we derived advantage from when developing an assay to find Gpr1 mutants with enhanced glucose affinity. The Gpr1 mutagenesis library generated various mutants with altered glucose sensitivity or maximum response to glucose. Hence, the new structural information will be essential in tuning the sensitivity of the mammalian circuit. Particularly, the amino acid in position 640 seems to be important for glucose interaction and the next step will be to explore all other variants at this position before integrating it in a mammalian signaling circuit.

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mutated activations sites of P_{SUC2} Fig (18).

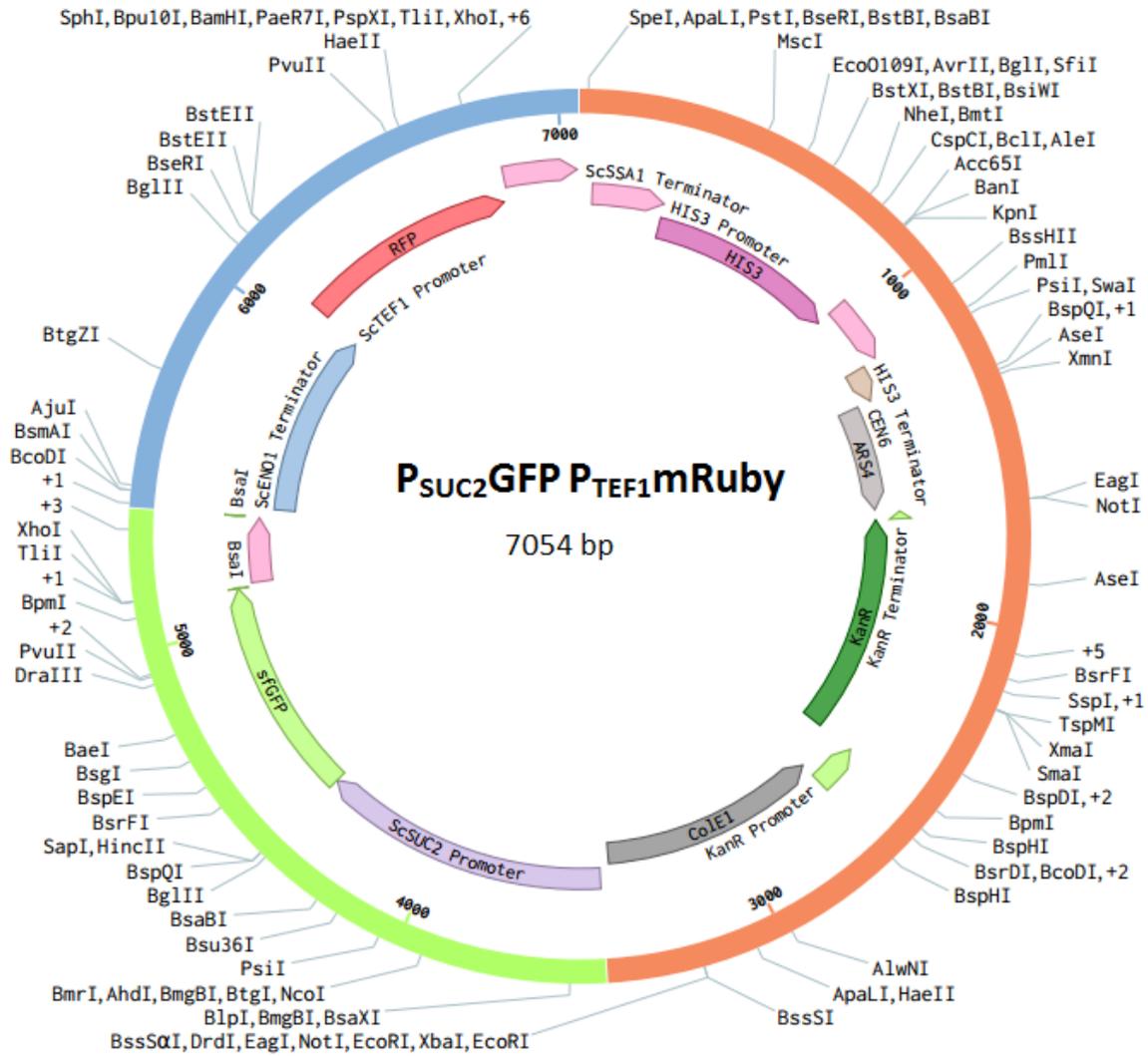


Figure 18: The plasmid containing the $P_{SUC2}sfGFP$ and $P_{TEF1}mRuby$ reporter assembled in the acceptor vector $pWSP037$ (low copy number), with the auxotrophic marker histidine.

A.2 Library generation constructs

The Gpr1 library was generated by mutate the Gpr1 and assemble on a plasmid (Fig 19).

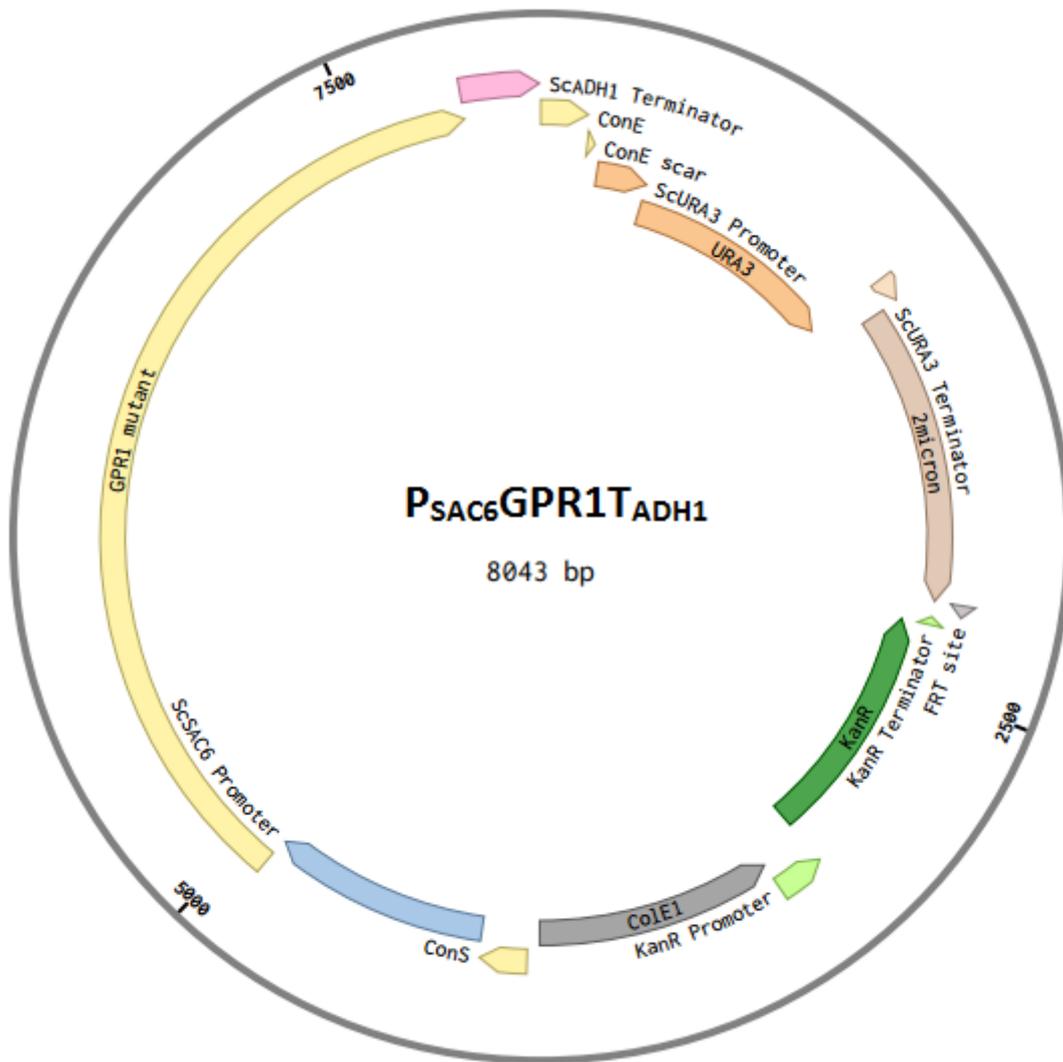


Figure 19: The plasmid containing the P_{SAC6} , the mutant $GPR1$ and the T_{ADH1} assembled in the acceptor vector $pWSP038$ (high copy number), with the auxotrophic marker uracil.

The $Gpr1$ mutants of interest were assembled on a plasmid for chromosomal integration in the URA -locus (Fig 20).

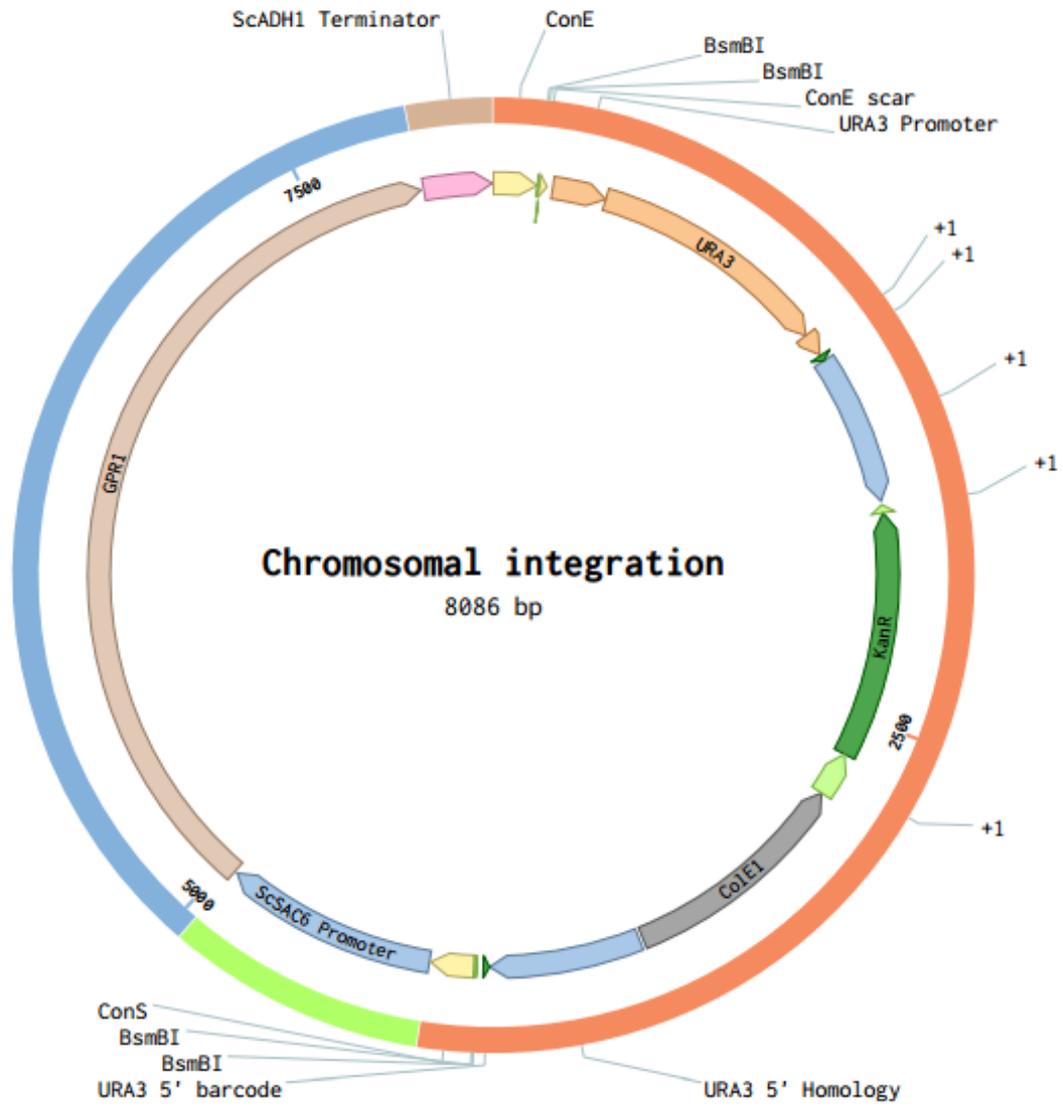


Figure 20: The plasmid containing the P_{SAC6} , the mutant $GPR1$ and the T_{ADH1} assembled in the $pYTK096-Ura$ integration vector, with the auxotrophic marker *uracil*.

The $Gpr1$ mutants of interest were assembled on a plasmid with a GFP-tag in the C-terminus (Fig 21).

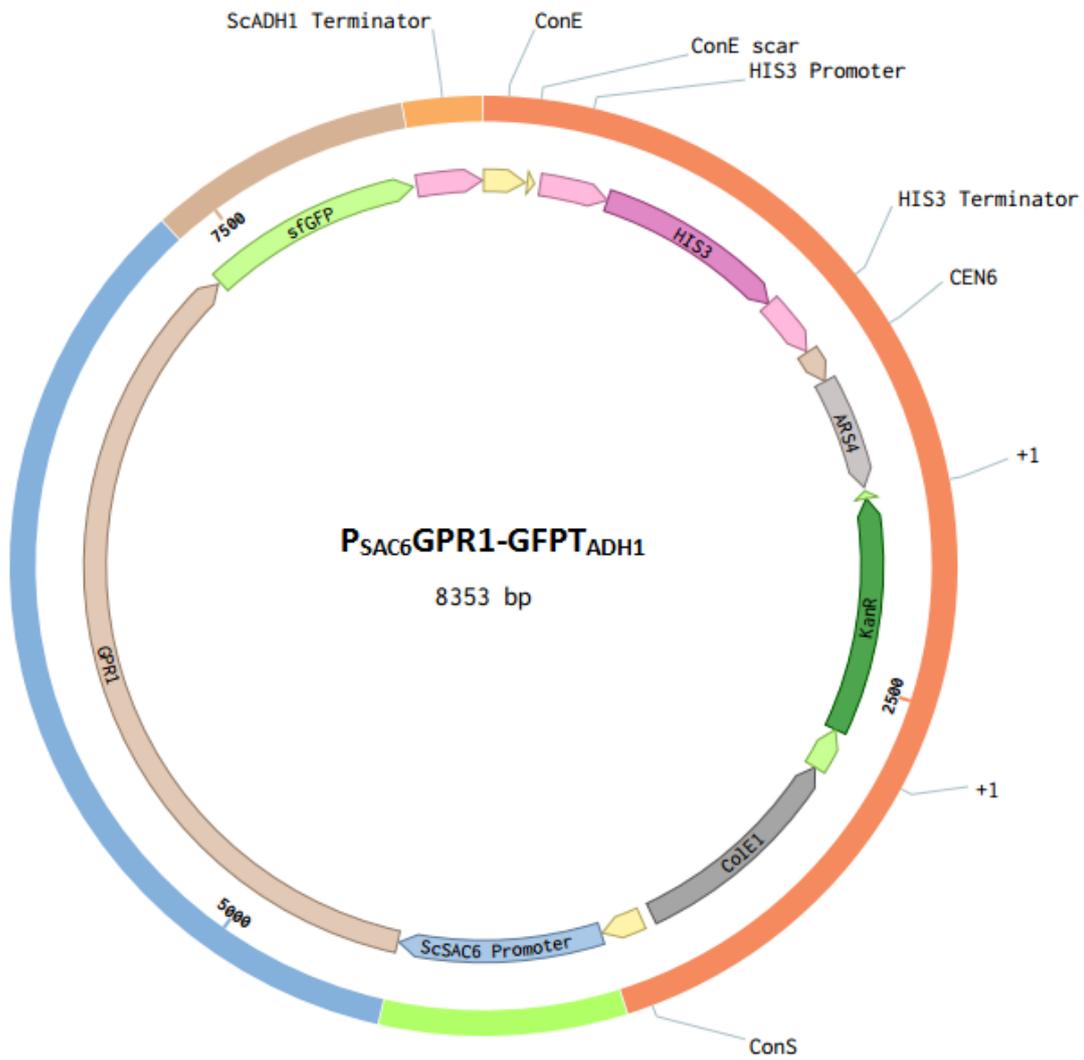


Figure 21: The plasmid containing the P_{SAC6} , the mutant $GPR1$ with a C-terminus GFP-tag and the T_{ADH1} assembled in the acceptor vector $pWSP037$ (low copy number), with the auxotrophic marker histidine.

A.3 Plasmids generated

All the plasmids generated in the project are concluded in Table 2.

Table 2: The plasmid constructed in the project. The different parts are from the yeast toolkit developed by Lee et al. 2015 [3].

	Description	Vector
Reporter strain	$pP_{SUC2sfGFPT_{SSA1}}$	pWSP037 (low-copy)
Reporter strain	$pP_{SUC2sfGFPTENO1P_{TEF1}mRubyT_{SSA1}}$	pWSP037 (low-copy)
Reporter strain	$pP_{SUC2AsfGFPTENO1P_{TEF1}mRubyT_{SSA1}}$	pWSP037 (low-copy)
Reporter strain	$pP_{SUC2BsfGFPTENO1P_{TEF1}mRubyT_{SSA1}}$	pWSP037 (low-copy)
Reporter strain	$pP_{SUC2ABsfGFPTENO1P_{TEF1}mRubyT_{SSA1}}$	pWSP037 (low-copy)
Mutagenesis library	$pP_{SAC6GPR1T_{ADH1}}$	pWSP038 (high-copy)
GFP-tagged Gpr1	$pP_{SAC6GPR1sfGFPT_{ADH1}}$	pWSP037 (low-copy)
Chromosomal integration Gpr1	$pP_{SAC6GPR1T_{ADH1}}$	pYTK096 (URA-locus)

A.4 Strains generated

All the strains generated in the project are concluded in Table 3.

Table 3: *Strains generated in the project.*

Background	Genotype	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Dharmacon
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gpr1Δ</i>	Dharmacon
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ras2Δ</i>	Dharmacon
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gpr1Δras2Δ</i>	Dharmacon
LL11	$P_{SUC2}LEU$ p $P_{SUC2}sfGFP$	This study
LL12	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2}sfGFP$	This study
LL13	$P_{SUC2}LEU$ <i>ras2Δ</i> p $P_{SUC2}sfGFP$	This study
LL14	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2}sfGFP$	This study
LL21	$P_{SUC2}LEU$ p $P_{SUC2}sfGFP$ $P_{TEF1}mRuby2$	This study
LL22	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2}sfGFP$ $P_{TEF1}mRuby2$	This study
LL23	$P_{SUC2}LEU$ <i>ras2Δ</i> p $P_{SUC2}sfGFP$ $P_{TEF1}mRuby2$	This study
LL24	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2}sfGFP$ $P_{TEF1}mRuby2$	This study
LL31	$P_{SUC2}LEU$ p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$	This study
LL32	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$	This study
LL33	$P_{SUC2}LEU$ <i>ras2Δ</i> p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$	This study
LL34	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$	This study
LL41	$P_{SUC2}LEU$ p $P_{SUC2B}sfGFP$ $P_{TEF1}mRuby2$	This study
LL42	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2B}sfGFP$ $P_{TEF1}mRuby2$	This study
LL43	$P_{SUC2}LEU$ <i>ras2Δ</i> p $P_{SUC2B}sfGFP$ $P_{TEF1}mRuby2$	This study
LL44	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2B}sfGFP$ $P_{TEF1}mRuby2$	This study
LL51	$P_{SUC2}LEU$ p $P_{SUC2AB}sfGFP$ $P_{TEF1}mRuby2$	This study
LL52	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2AB}sfGFP$ $P_{TEF1}mRuby2$	This study
LL53	$P_{SUC2}LEU$ <i>ras2Δ</i> p $P_{SUC2AB}sfGFP$ $P_{TEF1}mRuby2$	This study
LL54	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2AB}sfGFP$ $P_{TEF1}mRuby2$	This study
LL1Gpr1	$P_{SUC2}LEU$ <i>gpr1Δ</i> p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$ p $P_{SAC6}GPR1T_{ADH1}$	This study
LL2Gpr1	$P_{SUC2}LEU$ <i>gpr1Δras2Δ</i> p $P_{SUC2A}sfGFP$ $P_{TEF1}mRuby2$ p $P_{SAC6}GPR1T_{ADH1}$	This study
LL1Gpr1Ura	$P_{SUC2}LEU$ $P_{SAC6}GPR1$ <i>gpr1Δ</i>	This study
LL2Gpr1Ura	$P_{SUC2}LEU$ $P_{SAC6}GPR1$ <i>gpr1Δras2Δ</i>	This study
LL1GFP	$P_{SUC2}LEU$ p $P_{SAC6}GPR1sfGFP$ <i>gpr1Δ</i>	This study
LL2GFP	$P_{SUC2}LEU$ p $P_{SAC6}GPR1sfGFP$ <i>gpr1Δras2Δ</i>	This study

A.5 Primers

The primers used for amplification or verification are concluded in Table 4.

Table 4: *The primers used in the project.*

Primer	Sequence
URA3 5' forward	GGGCGGATTACTACCGTT
URA3 5' reverse	GTAATGTTATCCATGTGGGC
URA3 3' forward	AGAGCACTTGAATCCACTGC
URA3 3' reverse	GATTTGGTTAGATTAGATATGGTTTC
GFP4a forward	GCATTTCGGTCTCAATCCCGTAAAGGCGAAGAGCTG
GFP4a reverse	ATGCGGTCTCAGCCACTCGAGTTATCATTGTACAGTTCATCCATACC
EP-Gpr1 forward	CGTAGTCGGTCTCATATG
EP-Gpr1 reverse	GTCTGGTCTCAGGATCCTAATG
GPR1A640C forward	AGTCGGTCTCATTGCGATGCGTTGCAATAC
GPR1A640C reverse	GTCTGGTCTCCGCAAATGATGGGGAAAAGC
Sequencing primer 1	ACAAACAGCCGGTACAAG
Sequencing primer 2	TGTTGAGATGACAGCGAG
Sequencing primer 3	TGTGAGAAGGGATATGATTAC
Sequencing primer 4	CTACCACGGTTAACCAGC
Sequencing primer 5	ACGGCAGACGCAGTTATAG
Sequencing primer 6	ACAACCTTCCCGATTCTCC
Sequencing primer 7	CCAAGGTTTGTAGCCACC
Sequencing primer 8	ACAGTGCTAAACCACTCG
Sequencing primer 9	TGAGTGTGATGTCATTTTCAC
Sequencing primer 10	TCTCAACAATTTTCACAGGAG
Sequencing primer 11	TGCTGTTGTACGTTGGTG
Sequencing primer 12	CTGTGGTTTGTGTAACGC
Sequencing primer 13	CCCAGTATTCAAAATCGTCAC
Sequencing primer 14	CAAGCCCACAATACCAGG
Sequencing primer 15	GCTATCAAATCTATGCCGC
Sequencing primer 16	TGCCATATATGCAGTGGTG