Synthetic Cellular Signaling Circuits

Final Report
Publishable Summary

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Coordinator:	Prof. Horst Vogel, École Polytechnique Fédérale de Lausanne
Website:	synsignal.eu

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1 Executive Summary

Cellular signaling systems are crucially important for a broad range of critical health and disease areas and high value industrial applications. Signaling systems are the target for more than half of the medicines marketed by the pharmaceutical industry, and form the main Research & Development area for the nutrition, flavour and fragrance industries. SynSignal is a multidisciplinary high-tech consortium working in synthetic biology's area of greatest untapped potential, delivering a synthetic biology toolbox and finished products custom designed for major present and future industrial applications of cellular signaling.

Synthetic Cellular Signaling Circuits of interest for SynSignal. Natural cellular signaling cascades are comprised of multiple functional components, with each individual component of the system typically being a protein, or a multiprotein complex. Traditional biochemical and molecular biology methods focused on studying the individual component parts of signaling cascades in isolation, while more modern approaches including systems biology, genomics and proteomics have provided insight into the function of signaling cascades as a whole. Synthetic biology provides a powerful and new perspective - bringing additional methodologies and thought processes to bear- particularly classical engineering disciplines. From a synthetic biology perspective, synthetic cellular signaling circuits are perceived as being analogous to electronic circuits. Each component of the circuit, encoded by a DNA sequence of defined structure and function, is physically interchangeable with compatible modular building blocks of similar or dissimilar function, which makes the system on the whole designable and thus accessible for engineering. The cellular context of the signaling circuit, the "chassis", is considered as being similarly modular and designable. This way of thinking is beginning to provide powerful new tools and methods to understand, and more importantly *to control and modulate* the complex signaling systems of cells.

Industry and Cellular Signaling. Development times for new products across a broad range of industries that focus on cellular signaling are excessively long, with associated high costs. In addition, the lack of effective screening platforms for a number of high value industrial applications involving signaling has a severely adverse effect on innovation and discovery, impeding the delivery of new products. These deficiencies create an economic bottleneck that makes the search for promising technologies to overcome this bottleneck a high priority. Therefore, design of synthetic cellular signaling circuits is one of the key areas of development in synthetic biology, with a number of high value industrial applications, spread across several key European industry sectors. SynSignal addressed this urgent and imposing bottleneck, by providing new and sophisticated synthetic biology tools to overcome the challenges facing signaling-based product development in the pharmaceutical, flavour, fragrance and nutritional industries.

2 Summary description of project context and the main objectives

Cellular signaling systems are crucially important for a broad range of critical health and disease areas and high value industrial applications. Signaling systems are the target for more than half of the medicines marketed by the pharmaceutical industry, and form the main Research & Development area for the nutrition, flavour and fragrance industries. SynSignal was a multidisciplinary high-tech consortium working in synthetic biology's area of greatest untapped potential, delivering a synthetic biology toolbox and finished products custom designed for major present and future industrial applications of cellular signaling.
Cellular signaling is the sum of the communication system responsible for controlling most if not all cellular activities and for coordinating cell actions. Signaling systems allow the cell to perceive its environment and to respond to the information gathered in a manner which maximally benefits the whole organism. Signaling systems are crucial for chemosensory detection, efficient homeostasis, proper development, cellular repair, immunity, and for cognitive processes in higher organisms.
Errors in signal perception or processing cause severe human pathologies including cancer, diabetes, and immune disorders. In fact **cellular signaling systems are the target for more than half of the medicines marketed by the pharmaceutical industry**! Cellular signaling systems are also of crucial importance to several other **key European industries**. Chemosensory signaling systems present in our mouths and nose and gut allow us to perceive tastes, smells, and nutrients and therefore **chemosensory signaling systems are the central area of R&D in the nutrition, flavour and fragrance industries**. The discovery of taste and smell related signaling systems in non-chemosensory tissues such as the gut, lung, and pancreas present new therapeutic options for disease treatments, and reinforce the traditional link between nutrition and health. The importance of cellular signaling was recognized by the 2012 Nobel Prize in chemistry, awarded for key discoveries made by researchers in understanding the function of G-Protein Coupled Receptors (GPCRs).

Synthetic Cellular Signaling Circuits. Natural cellular signaling cascades are comprised of multiple functional components, with each individual component of the system typically being a protein, or a multiprotein complex. Traditional biochemical and molecular biology methods focused on studying the individual component parts of signaling cascades in isolation, while more modern approaches including systems biology, genomics and proteomics have provided insight into the function of signaling cascades as a whole. Synthetic biology provides a powerful and new perspective - bringing additional methodologies and thought processes to bear- particularly classical engineering disciplines. From a synthetic biology perspective, **synthetic cellular signaling circuits are perceived as being analogous to electronic circuits**, with the individual component parts functioning independently (orthogonally) from each other. Each component of the circuit, encoded by a DNA sequence of defined structure and function, is physically interchangeable with compatible modular building blocks of similar or dissimilar function, which makes the system on the whole designable and thus accessible for engineering. The cellular context of the signaling circuit, the "chassis", is considered as being similarly modular and designable. This way of thinking is beginning **to provide powerful new tools and methods to understand, and more importantly *to control and modulate* the complex signaling systems of cells**.

Industry and Signaling. Development times for new products across a broad range of industries that focus on cellular signaling are excessively long, with associated high costs. In addition, the lack of effective screening platforms for a number of high value industrial applications involving signaling has a severely adverse effect on innovation and discovery, impeding the delivery of new products. Taken together, these deficiencies **create an economic bottleneck** that makes the search for promising technologies to overcome

this bottleneck a high priority. Indeed, the European Academies Science Advisory Council (EASAC) policy report 13 (December 2010) states that **design of synthetic cellular signaling circuits is one of the key areas of development in synthetic biology**, with a number of high value industrial applications, spread across several key European industry sectors.
Based on the **leading expertise and innovative drive** of the partners, SynSignal addressed this urgent and imposing bottleneck, by providing new and sophisticated synthetic biology tools to overcome the challenges facing signaling-based product development in the pharmaceutical, flavour, fragrance and nutritional industries.

Essential Objectives of SynSignal are:
1. Implement a development program consisting of an iterative cycle of i) **Design & Engineering, ii) DNA Assembly and Protein Production, and iii) Testing** to accumulate a **toolbox** of synthetic parts (bio-bricks), cell lines (chassis), and complete signaling circuits.
2. To develop tools for our signaling toolbox with **broad combinatorial potential** applicable for different types of signaling cascades.
3. To develop whole synthetic signaling pathways that are applicable as **screening platforms for creating new medicines in key disease areas**, particularly **Cancer** and **Diabetes**.
4. To develop synthetic signaling cascades which are **transferrable into human cells for the treatment of specific diseases**.
5. To develop human signaling pathways are applicable as screening platforms for creating **new Flavour, Fragrance and Nutritional ingredients**.
6. To generate **technologies and intellectual property that is widely disseminated** to the broader European SME and large industry community.
7. To **identify societal perceptions and concerns about synthetic biology in Europe** in order to ensure long-term impact and sustainability of research efforts in the field.

Putting in place the **technological innovations, toolbox, and application-focused materials** created during SynSignal has an impact on the accessibility of drug discovery technologies, particularly for cancer and diabetes, and for discovery technologies to develop the next generation of flavours, fragrances, and nutritional ingredients. Our innovations provide immediate benefits to industry and the health of Europe's citizens and competitiveness of key European industries. In addition, all partners in SynSignal themselves drew great benefit from the activities proposed here. The private sector partners **enhanced their discovery and technology platforms**, thereby increasing their efficiency, output and valuation. Academic partners in SynSignal **advanced fundamental life science research** in their laboratories and in the larger scientific community by creating enabling tools to tackle biological questions of unprecedented complexity.

Through the already established relations between SynSignal members and large pharmaceutical enterprises in Europe, these major employers of European citizens benefited from our achievements, by integrating the novel technologies created in SynSignal into their discovery pipelines through **co-developments, licenses and future partnerships**. Similarly, SynSignal partners have longstanding ties with major European flavour, fragrance, and nutritional ingredient companies, and industrial dissemination of these technologies developed during SynSignal was a major focus of our Work Plan. Paramount objectives of SynSignal were to **ensure highest quality of research and development**, **innovative drive and effective dissemination of new technologies and methodologies**.

SynSignal reached out to the pan-European life science community in academia and industry, via focused networking activities. To achieve these goals, we have selected a unique combination of nine partners, including three **established and successful SMEs** (BioXtal, Geneva Biotech, Eurice), and six **leading academic research institutions** (École Polytechnique Fédérale de Lausanne, Institute for Human Nutrition, Karolinska Institute, Systems Biology Ireland, University of Bristol) which, as leaders in their respective fields, are at the forefront of technology development in the relevant areas for this proposal. A **major issue in synthetic biology regards societal perceptions of its relative safety, of ethical issues in its use, and of its potential effect on the environment**. Acceptance of this new technology by the general public is an area of major concern, comparable to the debate that circled around genetically modified food in Europe 10-20 years ago. SynSignal is approaching this problem through interaction with a dedicated team of experts in social science which **goes far beyond a narrow due diligence of our own technology development with a much more ambitious approach to find ways to reduce chances of public hostility toward synthetic biology as an emerging discipline**.

All SynSignal SME and academic partners were selected on the basis of **strict excellence criteria** in terms of quality of existing infrastructure, performance on the market, established track record in research, innovation and development and future plans. Together, SynSignal covers the entire domain of synthetic biology technology development, with **leading European experts in signaling system i) design and engineering ii) DNA assembly and protein production iii) testing**. From the onset, the SynSignal team already combined cutting-edge approaches in innovative technology development which are among the most advanced in their respective fields.

The complete work plan of SynSignal was divided into **eight Work Packages**. The eight Work Packages of SynSignal, in line with the **superior expertise** present in the team, were defined by the requirements of the field, in accordance with the **technological know-how** of the partners. In particular, our team's strengths lie in **modeling of signal systems, creation of synthetic signaling systems, systems biology, cutting-edge high throughput methods for multiprotein expression,** and **focused experience in chemosensory signaling, diabetes signaling, cancer signaling and GPCR signaling**.

Dissemination, innovation related activities and valorisation were among the paramount objectives of this consortium. The project outcomes were designed to massively **boost the standing of all partners**, SME and academic, in SynSignal. The tools and technologies prepared were valuable for advancing the research of the academic partners. The high-technology platforms created in SynSignal expanded patent portfolios and methods arsenals with immediate benefits. In summary, the activities of SynSignal were **highly targeted towards innovation of synthetic biology tools for a range of industry targeted signaling applications, roll-out of technologies and provision and valorisation of products**.

3 Description of main scientific & technological results/foreground

Work Package 2 – Computer Aided Design of Synthetic Signaling Systems

Main objectives of WP2

O2.1: Exploit mathematical modelling for the meaningful interpretation of the experimental data obtained in WPs 2-6 to quantitatively understand and predict the input-output response relationships, the spatiotemporal dynamics of signal propagation, and the emerging functional connectivities between protein components of synthetic signaling cascades.

O2.2: Design and mechanistically model synthetic dynamic signaling systems to reconstruct bistable, oscillatory and excitable behaviour of protein modification cascades.

O2.3: Create generally applicable open source software for designing and building synthetic signaling circuits. The synthetic parts and circuits created during SynSignal will be interfaced with the software, and SynSignal building blocks will be integrated as part of the design. Both software and building blocks will be made available to the wider synthetic biology community upon completion of the project.

Activities and results of WP2

Creating a generally applicable open source software for designing and building synthetic signalling circuits.

We have developed and delivered three software packages, which are generally applicable for designing, building, and studying synthetic signalling circuits: *Signalling Motif Generator*, *Dynamics Visualization based on Parallel Coordinates (DYVIPAC)* and *Parameter Estimation Pipeline for Systems and Synthetic Biology (PEPSSBI)*. All of these packages are open-source and publicly available:

1. Signalling Motif Generator (motifGenerator), available online at the address: https://bitbucket.org/andreadega/motif-generator
2. Dynamics Visualization based on Parallel Coordinates (DYVIPAC), available online at the address: https://bitbucket.org/andreadega/dyvipac-python. DYVIPAC is published as: L. K. Nguyen, A. Degasperi, P. Cotter and B. N. Kholodenko. DYVIPAC: an integrated analysis and visualisation framework to probe multi-dimensional biological networks. *Scientific Reports*, 5, Article number: 12569. doi:10.1038/srep12569, 2015.
3. Parameter Estimation Pipeline for Systems and Synthetic Biology (PEPSSBI), available online at the address: https://bitbucket.org/andreadega/systems-biology-compiler. PEPSSBI is published as: A. Degasperi, D. Fey and B. N. Kholodenko. Performance of objective functions and optimisation procedures for parameter estimation in system biology models // *npj Systems Biology and Applications* ***3***, Article number: 20 (2017), doi:10.1038/s41540-017-0023-2.

The developed set of software packages creates a pipeline for studying signalling pathways and developing synthetic signalling pathways. The design of a synthetic signalling pathway begins with the definition of a mathematical model that is biologically sound and that is predicted to produce the desired input-output relationship or property. This is achieved by defining the model structure, and by identification of model

parameters. To assist in developing the model structure, we have developed the motifGenerator software package that allows the user to define pathway modules and combine them automatically in a combinatorial way. The modules are specified as sets of reactions, where user-defined labels are assigned to biochemical species. The labels indicate possible ways to combine the modules and obtain automatically composed signalling pathway motifs. The software package can be used to generate automatically models for both initial model design and for automatic exploration of variants of existing designs.

For model parameterisation, we have developed two software packages: DYVIPAC and PEPSSBI. DYVIPAC was developed to support the step of parameter exploration (i.e. for the search of parameters that result in the desired property) by relating the systems parameters with systems dynamics through visualisation. DYVIPAC uses SBML models as input and explores the model parameter space. It then computes the stability analysis for each parameter set, thus identifying parameter sets for which the given model is capable of, for example, producing stable oscillations or multistability. The results are then visualised using a parallel coordinate interface.

For the model refinement, to achieve fully quantitative representation of the system we have developed PEPSSBI software package. PEPSSBI is a software package for automating the parameter estimation pipeline in systems and synthetic biology. PEPSSBI is designed to automate repetitive tasks, such as data normalisation, objective function definition and deployment on a computer cluster for efficient parallel computation.

As a result, the software packages we have developed cover all stages of theoretical investigation of intracellular signalling cascades, including synthetic signalling cascades: from the very idea of the system up to fully quantitative description. The software packages we have developed were successfully used within SynSignal for the study of intracellular cascades in the scope of current project (see below).

A refined mathematical model of the synthetic MP1/BXB-ER/p14 scaffolding system

The MAPK pathway is not a linear signalling pathway, but has branching points allowing crosstalk with other pathways. A number of scaffold proteins including KSR, IQGAP, Arrestin, and MP1 organize RAF and MAPK functions, serving as binding platforms and controlling the spatial and temporal aspects of the signalling flux. While these scaffolds were investigated in parallel to RAF over the years, the dynamic complexes they generate remain enigmatic. It is not clear how an upstream signal is propagated through the maze of scaffold-mediated signalling complexes. Scaffolds are notoriously difficult to work with because both overexpression and knock-down can lead to the disruption of the signaling complex, a phenomenon known as combinatorial inhibition.

Using the above-mentioned software packages and modelling suites, we specifically included scaffold proteins as crucial mediators of the MAPK signalling cascade in a refined MAPK model. We concentrated on switch-like behaviour both experimentally and computationally. For this, we have developed and refined a model of synthetic scaffolding signalling system in which scaffold brings about switch-like (“digital”) behaviour into initially graded-response (“analog”) system. For the experimental part, the BXB-ER construct as a backbone for this system. BXB-ER is a fusion protein containing the kinase domain of Raf-1 and the hormone binding domain of the oestrogen receptor. BXB-ER lacks five of the six ERK phosphorylation sites, rendering it resistant to negative ERK feedback regulation. Upon stimulation with tamoxifen, this construct is capable to activate the MAPK/ERK pathway, in a dose dependent manner, through a mechanism independent of growth factor receptors and Ras proteins. In order to analyse the effect of a scaffold on

MAPK activation and dynamics, BXB-ER was genetically fused to the scaffolding protein MP1 through a 5 Glycine linker. This new fusion protein, referred to as MP1-BXB-ER, now consists of the full length MP1, the kinase domain of Raf-1 (Raf-1 KD) and the hormone binding domain of the oestrogen receptor (ER-HBD). Under these conditions the binding of Tamoxifen to ER-HBD induces a conformational change that renders Raf-1 KD available to activate the MAPK/ ERK signalling pathway mainly through MP1. From a mechanistic perspective MP1 is able to bind MEK and ERK in an activation dependent manner. Furthermore, there is compelling evidence suggesting that under physiological conditions MP1 is in a tight complex with its adaptor protein p14. More importantly MP1-p14 interaction is necessary for the correct interaction of ERK with the MP1 complex. Therefore, we co-expressed MP1-BXB-ER with p14 and this partner was also included in our model. As expected, our experimental data indicate that in the absence of p14, the BXB-ER and MP1-BXB-ER constructs do not show any difference in their activation pattern. Our data suggest an increased sensitivity of MP1-BXB-ER for Tamoxifen when co-expressed with p14. This sensitivity saturates at the stoichiometry of p14:MP1-BXB-ER of about 2:1. Thus, the quantitative agreement between developed model of synthetic pathway and its experimental implementation was achieved.

A refined mathematical model for RAF kinase dimerization

We have refined the previously developed mathematical model of synthetic scaffolding system that induces activation of ERK pathway by including details of dimerization of RAF molecules to make the model generally applicable for the investigation of ERK pathway. The RAS/RAF/MEK/ERK pathway is pivotal for cell proliferation and survival and is frequently hyperactivated in tumours. Oncogenic mutations in the RAS genes are one of most frequent oncogenic mutations in cancer. Despite long effort at developing RAS inhibitors, there is still no clinically available drug. As a result, the development of inhibitors of the kinases downstream of RAS has become a hot topic in drug development. Clinically used RAF inhibitors are ineffective in RAS-mutant tumours, enhancing homo- and heterodimerization of RAF kinases, and leading to paradoxical activation of ERK signalling. Numerous mechanisms of RAF inhibitor resistance result in enhanced RAF dimerization and cannot be overcome by existing RAF inhibitors. A way to overcome resistance is the use of inhibitor combinations, but it is unclear how the best combinations can be chosen.

Using a combined experimental and computational approach, we have built a mechanistic ERK pathway model that integrates thermodynamics and kinetics of drug-protein interactions, structural elements, post-translational modifications and cell mutational status to faithfully predict inhibitor responses at the network level. The model has given unobvious predictions about optimal inhibitor combinations that suppress ERK pathway. For example, the model has predicted, that in some cases the most effective inhibitor combinations are combinations of inhibitors that target the same RAF kinase but in different protein conformations.

The developed model predicted a number of unexpected and hidden properties of network responses to different types of RAF inhibitors. The model suggests that synergy can emerge between Type I and Type II, as well as between Type I½ and Type II RAF inhibitors and predicts new ways of overcoming RAF inhibitor resistance in RAS mutant cells. Particularly, according to the model in cells with WT BRAF and oncogenic RAS mutation both combination of Type I and Type II, and combination of Type I½ and Type II RAF inhibitors synergistically inhibit ERK pathway. In contrast, in cells with both BRAF V600E and oncogenic RAS mutations, the model predicted that synergy effects for combination of Type I½ and Type II RAF inhibitors are much stronger than for any other combination of inhibitors.

In addition, the model allowed to compare efficiency of currently used combination of RAF and MEK inhibitors with the efficiency of combinations of two structurally different RAF inhibitors. The combination of two RAF inhibitors have shown strong synergytic effects namely in conditions, in which combination of RAF and MEK inhibitors does not demonstrate synergy effects.

To test model predictions, we experimentally measured responses of MEK/ERK signalling to different RAF inhibitor types and their combinations in melanoma cells bearing oncogenic RAS, BRAFV600E mutations, or both BRAFV600E and NRAS mutations. The level of MEK and ERK phosphorylation was measured by means of Western Blot, MESOSCALE and Luminex immunoassay kits. Our experimental results corroborated model predictions, showing that two RAF inhibitors ineffective on their own can robustly suppress ERK pathway when used in combination.

In summary, our results suggest a new principle of targeting the same kinase with two structurally different inhibitors that bind to different kinase conformations.

Publications related to the project

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3. Zhang et al. *ACS Nano*, 2017, Jan 24:11(1):249-257, DOI: 10.1021/acsnano.6b05356
4. Degasperi et al. *NPJ Syst Biol Appl.*, 2017, Aug 8:3:20, DOI: 10.1038/s41540-017-0023-2
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Work Package 3 – Synthetic Signaling Cascades in Taste Perception

Main objectives of WP3

O3.1: Establishment of cell lines with synthetic taste signaling cascades
O3.2: Identification of small molecules modulating taste and beta cell activity through taste receptors
O3.3: Miniaturization of synthetic taste receptor assays for simplified high throughput screening campaigns
O3.4: Development of novel drugs targeting beta cells

Activities and results of WP3

Taste is mediated by taste receptors in specialized cells on the tongue, recognizing thousands of molecules and initiating specific signalling cascades, eventually leading to taste perception in the brain. The receptors for sweet, umami (savoury) and bitter taste are so far best characterized and since their discovery they have been the targets of the flavour and nutrition industry trying to develop new compounds to enhance the desirable and mask the undesirable taste qualities of food and other products. Therefore, there is a huge need for reliable, sensitive and robust high-throughput assays to screen for novel taste-activating, taste-blocking and taste-modulating substances. In recent years, there is also a growing focus on the molecules interacting with taste receptors as potential new drugs, as taste receptors have been found in different organs and tissues throughout the body (including the gastrointestinal and respiratory tract, reproductive organs, heart, or brain as well as in different cell types such as pancreatic beta cells or white blood cells). Although still much research is needed to understand their role and mechanism of action in tissues outside of the oral cavity, the prospects of influencing important processes such as cell metabolism, nutrient uptake, hormone secretion or immunity and defence mechanisms via taste receptors are very promising.

The activities in this Work Package concentrated therefore on developing and improving tools used for the discovery of novel taste-modulating molecules and subsequently validating them in screening campaigns. Substances confirmed to interact with taste receptors were then tested for potential effects influencing beta cell activity.

In the course of the project, several new cell lines harbouring taste signalling elements were established. One strategy was to improve the sensitivity and signal amplitude of an existing assay, based on modified HEK293 cells (widely used immortalized human embryonic kidney cells). Since receptors for sweet, umami and bitter taste use G-protein-mediated signalling pathway, these cells possess a special G-protein, Gα16gust44. It was engineered to facilitate the coupling of taste receptors to a signalling cascade which induces changes in the level of calcium ions in the cells. Those changes can be monitored and correlated with changes in receptor activation.
By introducing further elements of the taste signalling pathway into the HEK293 cells, a greater sensitivity of the assay and a more robust signal were expected. Among several signalling elements tested, the biggest improvement was achieved for three HEK293/Gα16gust44 cell lines, each additionally engineered to produce one of the human bitter taste receptors: TAS2R31, TAS2R14 or TAS2R43, in a stable manner. Signals in the new cells were significantly higher than in the parental cell line with a transient, short-time receptor production, making them an excellent and reliable tool for screening assays.
Further cell lines were created from HEK293/Gα16gust44 cells by introducing calcium permeable ion channels. Inserting those channels in the cell membrane allow the flow of additional calcium ions from

outside the cells into the cytoplasm upon activation. Engineering these cells proved especially beneficial for several poorly performing TAS2Rs. In these cases, a significant increase in the calcium signal was observed in comparison to HEK293/Gα16gust44 cells without such channels.
Another approach involved the expression of cation channels that are not selective for calcium ions. When activated, cations such as Na^+ and K^+ enter the cell, leading to cell membrane depolarization and further signal propagation. The HEK293/Gα16gust44 cells modified to express such channels, which were created for the SynSignal project, can be used to develop an alternative assay, measuring changes in membrane potential instead of calcium level changes when coupled to taste receptors. Alternatively, the cells can be further developed to include downstream signalling elements reacting to membrane voltage changes.
Another strategy resulted in creating an assay for testing free fatty acid receptors, and putative fat taste sensors. The signalling cascades for the receptors have been successfully produced in mammalian HEK293 cells as well as in insect cells. Reliable high-throughput readout has been established and validated.

Using the existing and newly developed cell-based assays, several screening campaigns for novel taste-relevant compounds were conducted. They resulted in the identification of a number of new molecules showing taste-modulating effects. The most important findings are listed below:

1. An odorant compound found in lemon grass, (R)-citronellal, is able to block 2 bitter taste receptors responding to caffeine, thus decreasing its perceived bitterness (Suess et al., 2016).
2. It has been demonstrated that an artificial sweetener cyclamate is an effective blocker of bitter taste receptors activated by another artificial sweetener, saccharin, which explains a reduction in bitter off-taste for the blend of both sweeteners (Behrens et al., 2017).
3. Numerous new agonists for human bitter taste receptors have been found**,** among them substances from plants used in traditional Chinese medicine (Behrens et al., 2017)
4. Ligands were found for 21 out of 35 mouse bitter taste receptors. Ligand spectra of human and mouse bitter taste receptors have been compared and in many cases found to differ between orthologous receptors (i.e. related receptors in different species coming from a common ancestral gene). The results are crucial for any studies using mouse as a model organism in the field of taste, as well as pancreatic beta cell and other taste receptor-expressing cell physiology, where differences in ligand spectra for human and mouse orthologous taste receptors must be taken into account (Lossow et al., 2016).
5. Fatty acid enhancer candidates of both natural and synthetic origin have been identified in screens using fatty acid receptors.

Moreover, a test study has been conducted using chicken bitter taste receptor as a model. A combination of computer modelling with mutagenesis and functional experiments has been used to identify the bitter taste receptor's binding site and to perform a successful *in vitro* screen for its new agonists (Di Pizio et al., 2017). Such approach is very promising and can be employed also for human taste receptors.

Since several bitter taste receptors have been identified in pancreatic beta cells, their well-characterized agonists were tested on isolated mouse pancreatic islets to find possible effects on insulin secretion. However, no such effects could be observed, either by targeting the naturally occurring mouse bitter taste receptors or synthetically introduced human bitter taste receptors. These results strongly emphasize the need for further research on the mechanisms of action and a possible role of taste receptors in beta cells.

Publications:

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Lossow, K., S. Hubner, N. Roudnitzky, J. P. Slack, F. Pollastro, M. Behrens and W. Meyerhof. "Comprehensive analysis of mouse bitter taste receptors reveals different molecular receptive ranges for orthologous receptors in mice and humans." J Biol Chem., 2016

Behrens, M., K. Blank, and W. Meyerhof, *Blends of Non-caloric Sweeteners Saccharin and Cyclamate Show Reduced Off-Taste due to TAS2R Bitter Receptor Inhibition.* Cell Chem Biol, 2017

Behrens, M., et al., Bitter substances from plants used in traditional Chinese medicine exert biased activation of human bitter taste receptors. Chem Biol Drug Des, 2017

Di Pizio, A., et al., Ligand binding modes from low resolution GPCR models and mutagenesis: chicken bitter taste receptor as a test-case. Sci Rep, 2017. 7(1): p. 8223.

Work Package 4 – Synthetic Olfactory Perception Signaling Cascades

Main objectives of WP4

O4.1: Using combinatorial, high throughput multi-protein expression to establish complete synthetic signal transduction cascades for functional screening of olfactory receptors (ORs) in cultured cells. Understanding the interplay of these protein components for creating robust signaling pathways with increased sensitivity for coupling receptor activation to optical or electrical read-outs. Exploring the generic use of core principles (core protein elements) of synthetic cascades for more wide-spread applications in GPCR-based drug discovery and for cost-effective screenings.

O4.2: Nanoliter miniaturization and multiplexing of synthetic signaling cascades by producing (sub-) micrometre-sized cell-derived native vesicles to use them for odorant or drug screening.

O4.3: Establishing functional screening of ORs derived from non-sensory tissues for determining their role as potential medical targets for the treatment of important human diseases like diabetes and infertility.

Activities and results of WP4

Synthetic olfactory signaling cascades have been established in insect cells and mammalian cells with the following optical read-out systems: i) G-protein dissociation assays with optical BRET readout between the GPCR and G-protein ii) B-Arrestin association assays with optical BRET readout between the GPCR and Arrestin. These assays measure the signaling cascade proximal to the olfactory receptor (OR). To investigate distal signaling processes in multicomponent synthetic GPCR synthetic signaling cascades, olfactory receptors with G(alpha)olf, Gβ and Gγ subunits, adenylate cyclase III (AC3) together with an olfactory cyclic nucleotide-gated (CNG) channel composed of three types of subunits, CNGA2, CNGA4, and CNGB1 have been assembled. The increased intracellular concentration of cAMP after OR activation opens the CNG channel to produce a signal which is detectable optically via e.g. cAMP biosensors, and also produces a depolarizing current which is detectable by whole cell patch clamp recordings.
Structure based in silico screening with cellular assays have been combined for discovering compounds that activate common downstream signaling nodes of olfactory receptor signaling cascades. The major industrial application for such downstream activator molecules would be that they could potentially serve as fragrance boosters, or fragrance modulators, in fine fragrances (perfumes). We have successfully discovered new synthetic chemical activators of downstream signaling elements in olfactory signaling cascades.
This approach has been extended for screening active compounds for ORs existing in non-sensory tissues: cells like pancreatic beta cells and muscle cells. Screening large chemical libraries, we found (i) compounds which influence insulin production in pancreatic beta cells, and (ii) compounds which induce muscle cell differentiation. Both findings are of interest for future medical applications.

Towards miniaturization of assays for screening GPCR (olfactory- and non-olfactory GPCRs) signaling cascades, the following has been achieved. (i) Plasma membrane vesicles (PMVs) released from mammalian cells (cultivated cells, primary cells, bood samples) have been purified and investigated by optical microscopy, cryo-electron microscopy, mass spectrometry and electrical recording across nanopores. (ii) A new assay platform has been developed based on micrometer agarose beads covered with plasma membranes of mammalian cells. The method allows to monitor by fluorescence microscopy to detect directly the activation of GPCR and is suitable for large compound screening in a very generally applicable in

a highly miniaturized format with single-cell and single-molecule sensitivity. (iii) Mammalian cells, expressing heterologously olfactory receptors or human bitter taste receptors have been immobilized on micro-electrode arrays to follow agonist induced receptor activation by electrical impedance measurements. This label-free assay method is suited for compound screening in a highly miniaturized format.

Work Package 5 – Synthetic Signaling Cascades in Cancer

Main objectives of WP5

O5.1: Successful linkage of a human cancer related GPCR with the MAPK pathway in yeast
O5.2: Establishing successful transcriptional activation of yeast Ste12 protein in mammalian cells
O5.3: Successful demonstration of functionality of the synthetic pathway in mammalian cells, and its applicability as a drug discovery platform.

Activities and results of WP5

Engineering signalling pathways is a relatively unexplored area. Creating synthetic signalling cascades is one of the most challenging tasks in synthetic biology. The ability to reconstruct and analyse signalling cascades in orthologous systems allows to study their fundamental design principles. Mechanisms can be challenged and predicted using mathematical models that are designed specifically for such well-described and quantifiable signalling system. Synthetic signalling cascades have also great potential applications as tools, for example, to rationally alter cell and tissue responses, for creating biotechnologically and biomedically useful cellular devices and therapeutic tools, and, for drug testing. The overall, and very ambitious, aim of WP5 was to create the first complete synthetic signalling pathway in a mammalian cell line, and to then use the developed system as a drug screening platform. In order to achieve this challenging goal, the work plan was broken down into three main tasks and objectives:

(1) Linkage of a human cancer related GPCR with the MAPK pathway in yeast. The overall aim was to create a synthetic pathway combining the human GPCR CXCR4 with the yeast Ste2 signalling pathway and to demonstrate its functionality in the human HEK293 cell line. The first challenge was to find a good way of linking the upstream human receptor (CXCR4) to the upstream part of the yeast Ste2 cascade. Our first approach was generating a chimeric GPCR between CXCR4 (human) and Ste2 (yeast). This design was inspired by a previously published chimera of human GPCR Frizzled and the yeast GPCR Ste2 (Dirnberger and Seuwen, 2007). When working on the design to identify the boundaries of the chimera based on sequence analyses, we already realized that there is only very limited structural homology between the two GPCRs. After extensive manual inspections, it became obvious that the sequences are too different to design a functional chimera. Indeed, when we experimentally tested one designed chimera, only few cells expressed the chimera. Moreover, in mammalian HEK293 cells the chimera was only expressed in few cells in inclusion bodies without membrane localization.

Second, we engineered a chimera between the human Galpha Gai that connects to CXCR4 and the yeast Galpha Gpa1 of the Ste2 cascade as described in the literature (Sachpatzidis et al., 2003; Klein et al., 1998). The chimera was generated by replacing the 5 C-terminal residues of the yeast Galpha (GPA1) by those of the human Galpha. This chimera worked correctly in yeast. Manual inspection of the X-ray structure of a GPCR in complex with Galpha, beta and gamma suggested, however, that the C-terminal helix of the Galpha subunits inserts more than 5 residues into the GPCR. Thus, another Galpha chimera was generated by replacing the last 13 C-terminal residues of the yeast Galpha. This chimera turned out to be constitutive active in yeast, and could serve as a positive control when testing the functionality of the whole pathway. To prevent cross-talk of the Galpha Gpa1(5) with other human GPCRs present in HEK293 cells and to prevent that CXCR4 only signals to the Galpha Gpa1(5), we next aimed to engineer mutations in the interface of the protein. The idea was to introduce compensating mutations in the interface of the two proteins, for example by introducing a bulky amino acid in one protein and that would introduce steric

clashes with all endogenous partners, and introduce a compensatory mutation (a small amino acid; predicted using the protein design algorithm, FoldX (Schymkowitz et al., 2005; Van Durme et al., 2011), which would enable only binding to the partner protein with the bulky residue. However, because we also wanted not to compromise folding of the mutant protein variants, we restricted ourselves to only allow mutations that have been seen already naturally occurring in any GPCR or Galpha protein. Based on these constraints, we could not design a mutation that matched both criteria. Therefore, the next idea was to generate a (long) linker between CXCR4 and Gpa1(5), which would artificially bring the two proteins together, but still enable dissociation of the Galpha from the GPCR once activated. This should minimize crosstalk. Manual inspections suggestion that the C-terminus of CXCR4 and the N-terminus of Gpa1(5) are close in space and adding a linker there could work. We have designed and cloned this linker-chimera and we tested its functionality in yeast, which is based on a published protocol (Dong et al., 2010), and had been successfully implemented in the lab before to show functionality of the Ga-chimera. The assay principle is that yeast cells are grown in medium lacking essential histidine and they can only produce histidine when the pathway is activated by the CXCR4 ligand SFD1a. Because the assay is leaky and some enzyme to produce Histidine (the His3 enzyme) is already made without the pathway being active, a competitor inhibitor of the His3 enzyme (3'AT) is added at 1-5 mM concentration (Dong et al., 2010). The 3'AT inhibitor needs to be carefully titrated: (i) not too low, which would cause growth without SDF1 addition and (ii) not too high, which would cause inability to grow even when SDFa1 ligand is present and the pathway active. Despite several repetitions of the assay and careful titration with 3'AT we could not get a reliable readout anymore using this assay. In the future, we will integrate the two linker chimeras into the MultiPrime modules (see (3)) and test their functionality within the context of the whole synthetic pathway in HEK293 cells.

(2) Transcriptional activation of yeast Ste12 protein in mammalian cells. Transcriptional activation in both yeast and mammalian is complex and involves aside from RNA polymerase and transcription factors, additional factors and mediators, such as the VP16 activation domain that interacts with multiple transcriptional components (Hall and Struhl, 2002). Fusion proteins of VP16 and transcription factors are frequently used tools in synthetic biology to design eukaryotic transcriptional circuits (Khalil et al., 2012). We designed an initial transcriptional circuit that expressed the reporter luciferase under the control of the Fus1 promoter in response to binding of the fusion protein Ste12-VP16. To test the dynamic range of luciferase expression, we expressed Ste12-VP16 in a doxycycline (Dox)-dependent manner. To avoid co-transfection of multiple plasmids, we used the 'ACEMBL' systems developed in I. Berger's lab (partner in SynSignal). The 'ACEMBL' system to enable fast and flexible generation of multigene delivery constructs by an automatable technique called tandem recombineering (TR) to combine 'Donor' and 'Acceptor' plasmid modules via reversible Cre-loxP fusion in vitro (Bieniossek et al, 2009). This system was then modified in collaboration between the I. Berger and L. Serrano labs to include donor vectors that express genes in a controlled (doxycycline-dependent) manner ('TEMTAC', Beltran-Sastre et al, Sci. Rep., joined publication). Expression of Ste12-VP16 in a Dox-dependent manner resulted in increased luciferase activities compared to the expression of Ste12 alone. This suggested that the VP16 domain is indeed needed to achieve transcription in mammalian cells with a yeast transcription factor. However, luciferase activities were close to the experimental error/noise, suggesting that the dynamic range is very small. One possible reason could have been that the rather long sequence of the Fus1 promotor binds unspecific to other mammalian transcription factors, regulators, etc. Therefore, to increase the dynamic range, a new synthetic minimal promoter was designed and tested. This synthetic promotor contains part of the luc2 sequence and promotor from pGL4.26 (Promega). pGL4.26 contains a minimal promoter where upstream response

elements can be inserted. For our purposes, we inserted four PRE sites from the Fus1 promoter and two additional PRE sites from the Ste12 promoter. Expression of Ste12-VP16 in a Dox-dependent manner using the new synthetic minimal promoter resulted in a 5- to 10-fold increased dynamic range of luciferase activities compared to the expression of Ste12 alone or the control plasmid.

Having demonstrated functionality of the basic transcriptional machinery, we then worked upwards to test the ability to regulate transcription through upstream signalling events. We have shown that co-expression of the Ste12 inhibitors Dig1 and Dig2 reduced the luciferase expression, as expected. Since then, we have tested the transcriptional circuit using by mimicking upstream phosphorylation events by co-transfecting phosphomimetics mutants of Ste12 (S400D, T525E), Dig1 (T277E and S279D and T280E and T277E, S279D, T280E), and Fus3 (T180D, Y182E), but none of the mutants affected the luciferase signal. We believe that we can only achieve proper regulation when the full signalling cascade is implemented, and proteins are naturally phosphorylated (possibly at multiple sites).

(3) Functionality of the synthetic pathway in mammalian cells, and its applicability as a drug discovery platform. The protein expression and quantification of the full cascade in HEK293 cells contains several challenges: (i) the expression needs to be at the right level as too high levels my cause toxicity; (ii) the detection of proteins using protein-specific antibodies, tagged proteins (e.g. flag tag), and/or mass spectrometry; and (iii) the quantification of expressed proteins requires targeted mass spectrometry with spiked-in heavy labelled peptides od known concentration. The successful demonstration of functionality of the synthetic pathway in mammalian cells, and its applicability as a drug discovery platform has, unfortunately, not been fully achieved to date. However, great progress has been made in designing, cloning, and testing a modular pipeline in collaboration with I. Berger using his newly developed tool, MultiPrime (Mansouri et al., 2016). In order to implement the full Ste2 cascade in HEK293 cells, three modules were designed (a GPCR module, a scaffold module, and a transcription module), which are first tested independently before testing them together. We used constitutive promoters for all constructs, except for the Ste5 scaffold, which is Tet inducible (but with a CMV promoter to ensure we can express it high). We used a combination of strong (CMV) and medium strong (PGK) promoters. All modules are (nearly) cloned by now (one is about to be finished), and the transcriptional module has been already tested. Similar as before, the yeast transcription factor Ste12 was fused to VP16, a transcriptional activation domain that recruits the mammalian transcription machinery (Ste12-VP16). Expression of Ste12_VP16, Dig1 and Dig2 is under a constitutive promoter and the phosphomimetic of Fus3 (T180D/Y182E 'active' mutants) was expressed in a doxycycline (Dox)-dependent manner. As observed before, co-expression of the repressors Dig1/Dig2 strongly reduced the normalized luciferase signal. However, this inhibition was not relieved by expression of a constitutive active Fus3 phosphomimetic. This cannot be due to lack or drastically different protein expression levels (all proteins were detected at similar levels by mass spectrometry). It is known from the literature that active Fus3 phosphorylates Ste12, Dig1, and Dig2, thereby causing a release of the inhibition, which is expected to increase the luciferase signal to the one without Dig1/Dig2. It is likely that the phosphomimetic of Fus3 is not working, and upstream activation by Ste7 is needed. However, in principle this module should work once it is hooked up to the full cascade.

We could not start with the screening of ligands as we have not achieved demonstrating the full functionality of the complete synthetic signalling cascade. Both the Luis Serrano Lab and the Imre Berger lab have a great commitment in achieving the tasks and, once SYNSIGNAL is finished, using internal lab resources. Therefore, our intention is to finish the network, patent it and look for companies interested to use it for screening.

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Work Package 6 – Synthetic Signaling Cascades in Diabetes

Main objectives of WP6

O6.1: Establishment of an in vivo screening platform for the functional assessment of synthetic signaling cascades in beta cells

O6.2: Improvement of beta cell function by stimulation of synthetic signaling pathways in engineered beta cells

O6.3: Improvement of blood glucose homeostasis by transplantation of beta cells engineered to express synthetic signal transduction pathways

Activities and results of WP6

The overall objective of WP6 was to establish and use *in vitro* and *in vivo* methodologies for the screening of novel ligands and synthetic signalling pathways based on GPCR activation for the improvement of beta cell function and/or proliferation.

We have established the required methodologies for the evaluation of synthetic signalling pathways in diabetes. The two most important readouts for improvement of beta cell function are based on (i) beta cell proliferation (leading to an increased in beta cell mass, thus to an increase overall potential to secrete insulin), and beta cell activation (leading to the essential secretion of insulin). Particularly, these readouts were applied to an *in vivo* imaging approach allowing the longitudinal assessment of beta cell function and replication in the living organism, which is required for the *in vivo* assessment of specific signalling pathways in native and engineered beta cells. We have previously established an *in vivo* imaging platform based on the transplantation of islets into the anterior chamber of the eye (Speier et al, 2008, Nat Med 14:574–578). These islet grafts can be imaged longitudinally by laser scanning confocal microscopy through the transparent cornea of mice under anesthesia. For this project, we have improved our *in vivo* transplantation and imaging platform for functional investigations of the implementation of synthetic signalling pathways in beta cells (Objective 6.1).

We have tested the fluorescent biosensor “fucci” (Sakaue-Sawano et al, 2008, Cell 132:487–498) and shown that it is a valuable tool for the assessment of beta cell proliferation both *in vitro* and *in vivo*. It is based on the specific emission of a fluorescent protein in the nuclei of proliferating cells, which fluorescent wavelength and intensity are adapted for imaging by laser scanning confocal microscopy.
Since an increase in intracellular calcium ($[Ca^{2+}]_i$) in beta cells triggers insulin release, $[Ca^{2+}]_i$ can be seen as a perfect readout for beta cell activity. We have assessed GCaMP3, an engineered protein that reports on changes in $[Ca^{2+}]_i$, and demonstrated that it can serve as a valuable tool for the assessment of beta cell activation both *in vitro* and *in vivo*. We show that an increase in $[Ca^{2+}]_i$ following glucose uptake can be monitored by an increase in GCaMP3 fluorescent intensity, which fluorescent wavelength and intensity are also perfectly adapted for imaging by laser scanning confocal microscopy.
These two biosensors are useful for the assessment of functional modulation induced by the expression of synthetic signalling pathways in beta cells (Objectives 6.2 and 6.3).

To study physiological effects of beta cells engineered to express synthetic signaling pathways, we developed and optimized a strategy to completely “replace” the pancreatic islets by engineered islets. This was achieved by chemical ablation of *in situ* pancreatic islets using either streptozotocin or diphtheria toxin, combined with the transplantation of intact islets into the anterior chamber of the eye. We have studied

the kinetics of beta cell destruction via these two strategies and established experimental conditions optimal to destroy *in situ* endogenous pancreatic endocrine cells. Chemical ablation of *in situ* pancreatic islets by diphtheria toxin in mice expressing the diphtheria toxin receptor (DTR) specifically in beta cells (Thorel et al, 2010, Nature, 464: 1149-54) induces hyperglycemia within a few days after diphtheria toxin treatment, showing a dramatic decrease in their pancreatic beta cell mass 15 days after treatment. Based on these experiments we have published a manuscript detailing the precise kinetics of loss in functional beta cell mass in this specific mouse model (van Krieken et al., 2017, Scientific Reports 7: 12440). The transplantation of approximately 100 pancreatic islets from a donor mouse into the anterior chamber of the eye of the recipient mice rendered diabetic by ablation of endogenous pancreatic beta cells was sufficient to normalize blood glucose levels, indicating a proper function of the islet grafts and a perfect "replacement" of the original endogenous pancreatic islets by islets transplanted into the anterior chamber of the mouse eye. This is essential for the assessment of engineered islets transplanted into the anterior chamber of the eye (Objective 6.3).

We have established screening methodologies for testing the activation of endogenously-expressed V1b receptors in beta cells. More specifically, we are able to assess *in vitro* the activation of this receptor by the induced increase in intracellular [Ca^{2+}], and by the subsequent release of insulin from isolated pancreatic islets at various glucose concentrations. Additionally, our approach permits to determine the concentration dependence of specific ligands for the V1b receptor. This screening methodology was complemented by an *in vivo* transplantation/imaging procedure, allowing to confirm that a ligand-mediated *in vitro* activation of the V1b receptor can also be observed under *in vivo* conditions. This complete set of *in vitro* and *in vivo* procedures directly serves the goals of WP6, by allowing to assess novel ligands or novel GPCRs for their influence on pancreatic islet function.

With the aim of probing the specificity of ligands for the targeted V1b receptor, we have designed and produced an adenovirus carrying the receptor's sequence under the insulin promoter. Overexpression of this synthetic construct in mouse islets lead to an increased function in terms of $[Ca^{2+}]_i$ increase and insulin release induced by ligands of the V1b receptor. In addition to allowing the assessment of ligand specificity for the V1b receptor, this strategy serves as a proof-of-concept reaching the main goal of WP6, which is to synthetically engineer pancreatic islets for their improved function.

We have tested a nanobody specific for the V1b receptor for its potential effect on V1b receptor activation. Our results demonstrate that this nanobody induces an increase in insulin release from mouse beta cells in a dose-dependent manner, via the activation of the V1b receptor. The specificity of the ligand-receptor couple was further demonstrated by the activation-induced internalization of the human V1b receptor. This novel ligand thus binds to the V1b receptor and acts as an agonist, confirming that nanobodies can be developed to target specific GPCRs and can possibly serve as ligands, serving as interesting molecular tools for other scientists within or outside the SynSignal consortium. Finally, we showed that this specific nanobody can activate islet beta cells *in vivo* by inducing an increase of intracellular [Ca^{2+}] upon intravenous administration in mice (Objective 6.2). Importantly, this is the first report of a nanobody-based ligand that can modulate pancreatic islet function, paving the way for the use of this family of molecules in the regulation of various biological processes.

In order to increase the gene transfer efficiency allowing to promote specific synthetic signalling pathways in beta cells within islets, we have established a technique allowing the adenovirus-mediated transduction of pseudoislets engineered from disaggregated mouse islet cells. These pseudoislets could be precisely

engineered for a control of their size and content. We demonstrated the very high efficiency of the synthetic human V1b receptor expression in pseudoislets that lead to an optimized signalling pathway in mouse beta cells. Furthermore, we could show that pseudoislets maintained their normal function in terms of glucose-induced insulin secretion *in vitro* and in terms of maintenance of proper blood glucose levels *in vivo* after metabolic transplantation of pseudoislets into the anterior chamber of the mouse eye in absence of endogenous *in situ* pancreatic islets.

To assess the preservation of synthetic expression over time in transplanted islets, we developed a methodology for gene expression analysis on single islet grafts. Amplified cDNA from islet grafts display characteristic expression patterns that are similar to islets isolated from the pancreas. By the genetic analysis of islets grafts dissected out from the transplantation site we could demonstrate that synthetic signalling pathway elements implemented into engineered beta cells have a maintained expression over a period of at least 4 weeks *in vivo*. This is necessary in order to assess the functional and physiological role of engineered islets, since the synthetic signaling pathways implemented into islet cells need to be present at the time of *in vivo* assessment. Using this methodology, we could overexpress the human V1b receptor in mouse beta cells and prove that the expression is maintained *in vivo*. By combining this with the GCaMP3 biosensor for $[Ca^{2+}]_i$ readout we could image the activation of this synthetically expressed receptor *in vivo* in the anesthetized mouse, and demonstrate that this activation occurs using a novel nanobody-based ligand for this receptor. This ligand furthermore led to an increase in insulin secretion upon *in vivo* administration, showing that the synthetic overexpression of this receptor led to an improved activation of its downstream signalling pathway *in vivo* (Objective 6.3).

Overall our results successfully complete the achievement objectives of WP6, and demonstrate the potential to improve beta cell function *in vivo* by the synthetic implementation of GPCR signal transduction pathway elements in islets prior to transplantation.

Work Package 7 – Functional Antibody Fragments to Stimulate or Inhibit Synthetic Signaling Cascades

Main objectives of WP7

O7.1: To discover and validate functional antibody fragments with potential direct therapeutic applications.

O7.2: To validate the synthetic signaling pathways and their associated receptors as targets for drug discovery in cancer and diabetes.

O7.3: To provide positive controls to demonstrate specific activity of the synthetic signaling systems developed in WPs 3-6

Activities and results of WP7

Activities in this work package have been dedicated to the discovery and characterization of functional antibody fragments targeting GPCRs and pathway proteins involved in cancer and diabetes, including establishment of mammalian stable cell lines, expression/purification of selected targets, immunization of llamas and library cloning from their antibody's repertoire, selection of antibody fragments by phage display, initial binding characterization by Elisa and FACS, cloning and large scale expression of positive antibody fragments and their functional characterization.

The major challenge for the generation of antibodies against membrane receptors is to obtain a very good over-expression of the target of interest to be able to obtain suitable yield for the immunization step. Starting from an initial list of 31 GPCR targets and 40 pathway targets potentially involved in cancer and diabetes, our researchers have successfully established stable cell lines for 30 GPCR target as illustrated in figure 1.

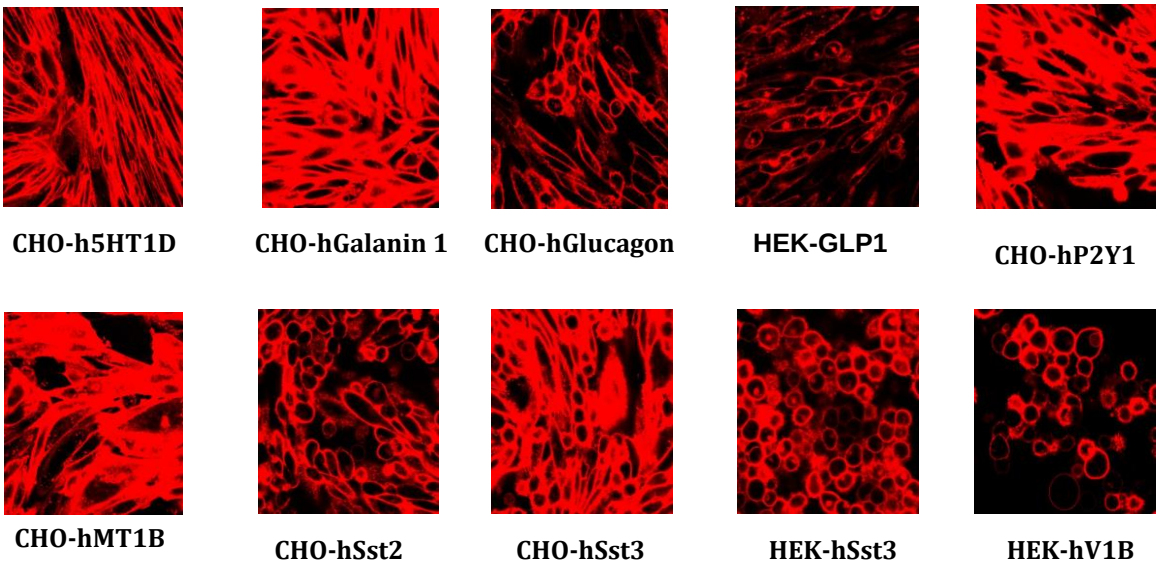


Figure 1: Confocal imaging of 10 GPCRs of the Synsignal target list

The second challenge for this work package relates to the ability of our researchers to scale-up and purify GPCRs at mg quantity. Expression of 8 receptors has been scaled-up in 20 roller bottles; plasma membranes have been prepared by ultracentrifugation after cell disruption. The GPRCs have been extracted from the

membrane using DDM detergent and purified on a Streptactin affinity column. Purification quality has been systematically assessed by SDS PAGE as illustrated in figure 2.

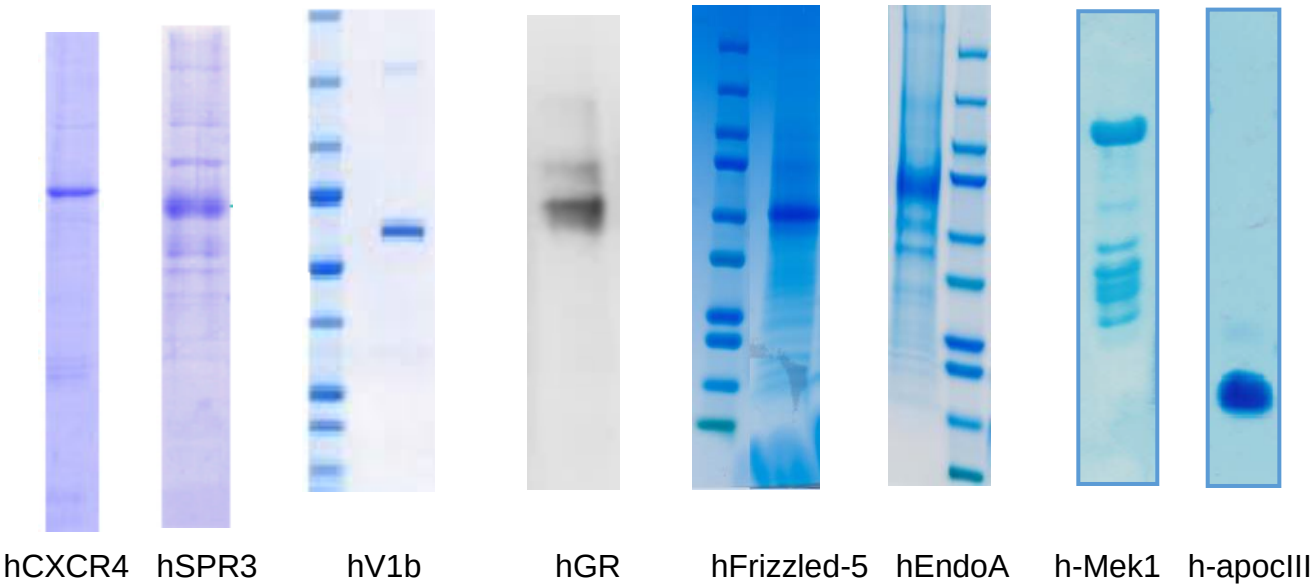
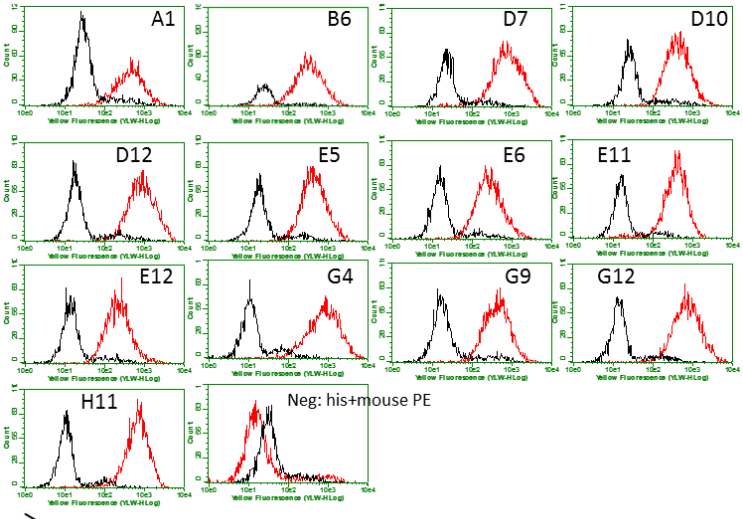


Figure 2: 6 GPCRs purity assessed by SDS PAGE and 2 soluble targets

The purified receptors and pathway targets have been used for immunization of llamas doing 6 weekly injections prior to the animal blood collection 5 days after the last injection.

Antibody fragment genes have been amplified from immunized animal PBMCs' isolated RNA by RT PCR and subcloned into a phagemid vector in order to generate a VHH phage library.

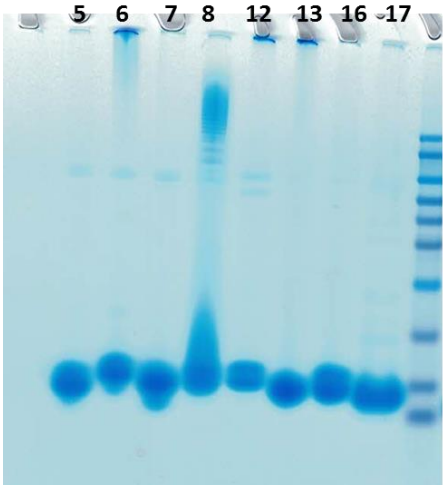
VHH have been selected by phage display on receptors (EndoA, S1P3, FDZ5, V1b, GR, CXCR4, LPAR2, LPAR6, P2Y10, 5HT2C) and pathway targets (RhoA, P115, ApocIII and Mek1) immobilized by direct coating on Nunc plate. After two rounds of selections, 96 clones have been picked-up and characterized by Phage Elisa. Elisa positive clones have been sent to sequencing and unique sequence clones further characterized by FACS as illustrated hereunder for the EndoA receptor.



→ ***Anti-his (1/200) + Mouse PE (1/200)***

Figure 3: Illustration of FACS analyses of several Elisa positive clones on EndoA receptor cell line.

All the FACs/Elisa positive clones have been sub-cloned into expression vectors and expressed in E.coli at mg yield quantities in order send them to the teams in charge of functional characterization in cellular and in vivo models.



5 : 13,8 mg
6 : 19,8 mg
7 : 19,2 mg
8 : 8,3 mg
12 : 4 mg
13 : 39 mg
16 : 36 mg
17 :1,7 mg

Figure 4: Illustration of SDS page gels performed on 8 anti hS1P3R purified VHH

For some expressed VHH, affinity curve have been determinated on various constructs showing nanomolar range for all the antibody fragments (on anti ApoCIII in the illustration here under).

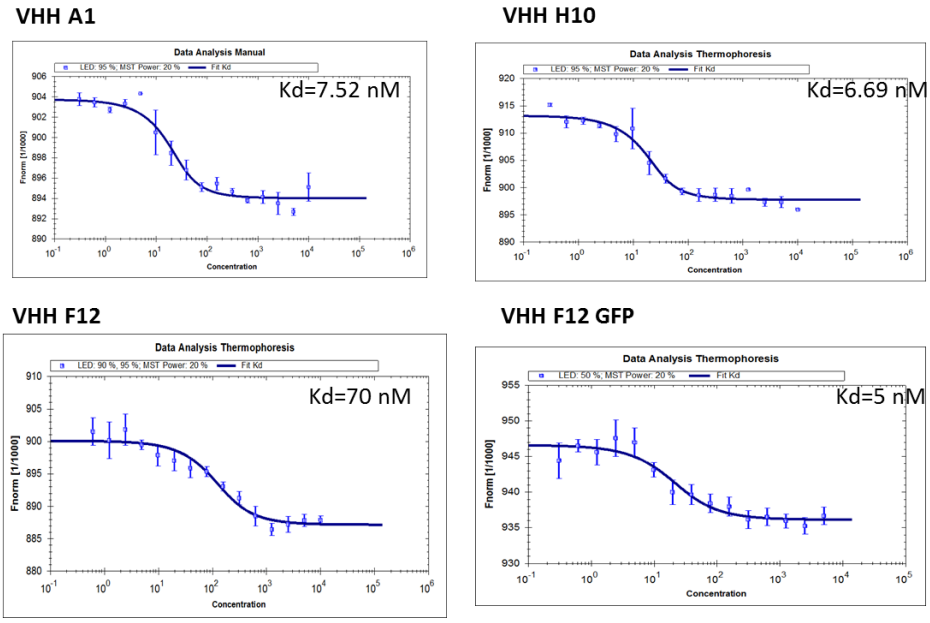


Figure 5: Affinities measured by thermophoreses on 4 different anti-ApoCIII VHHs

Among the VHH selected against GPCRs, 3 of them targeting the V1b receptor have been characterized in various functional assays including inositol phosphate pathway and Erk1-2 phosphorylation pathway.

Dose response curves performed with VHH10, 11 and 23 confirmed the agonist effect of VHH10 with an EC50 of 18.8 ± 1 (figure 6a). In the contrary, no activation could be obtained with the VHH 11 and 23 (figures 6b and 6c). Again the maximum IP1 concentration obtained with the VHH10 is equivalent to the

one obtained with AVP, leading to the conclusion that the VHH10 is a full agonist of the inositol phosphate pathway.

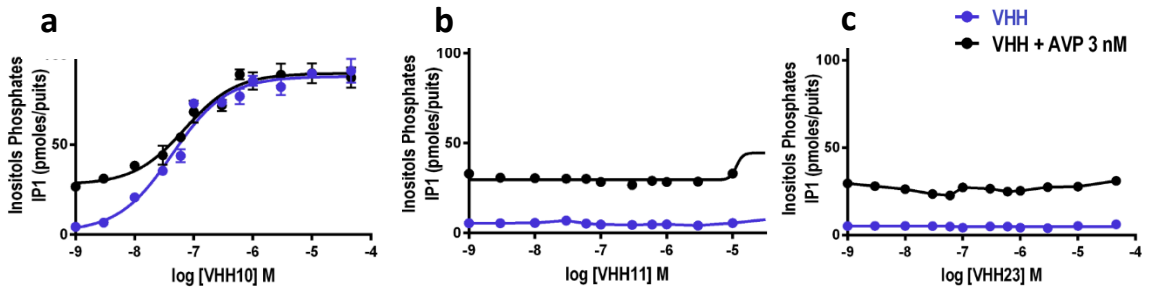


Figure 6: Pharmacologic properties of three VHHs on the hV1bR inositol phosphate pathway in a dose dependent manner. Dose response Curve of the VHHs alone in blue at the indicated concentration and the VHH in addition to a fixed concentration of AVP at the EC50 (EC50 = 3 nM) in black have been performed for the VHH10 a), VHH11 b) and VHH23 c). Error bars represent standards error of n = 3 measurements.

The induced internalization of the V1B receptor was also confirmed in living cells by fluorescence microscopy after the cell surface-expressed receptors were labelled with the cell-impermeable Streptactin Red fluorescent substrate (figure 7)

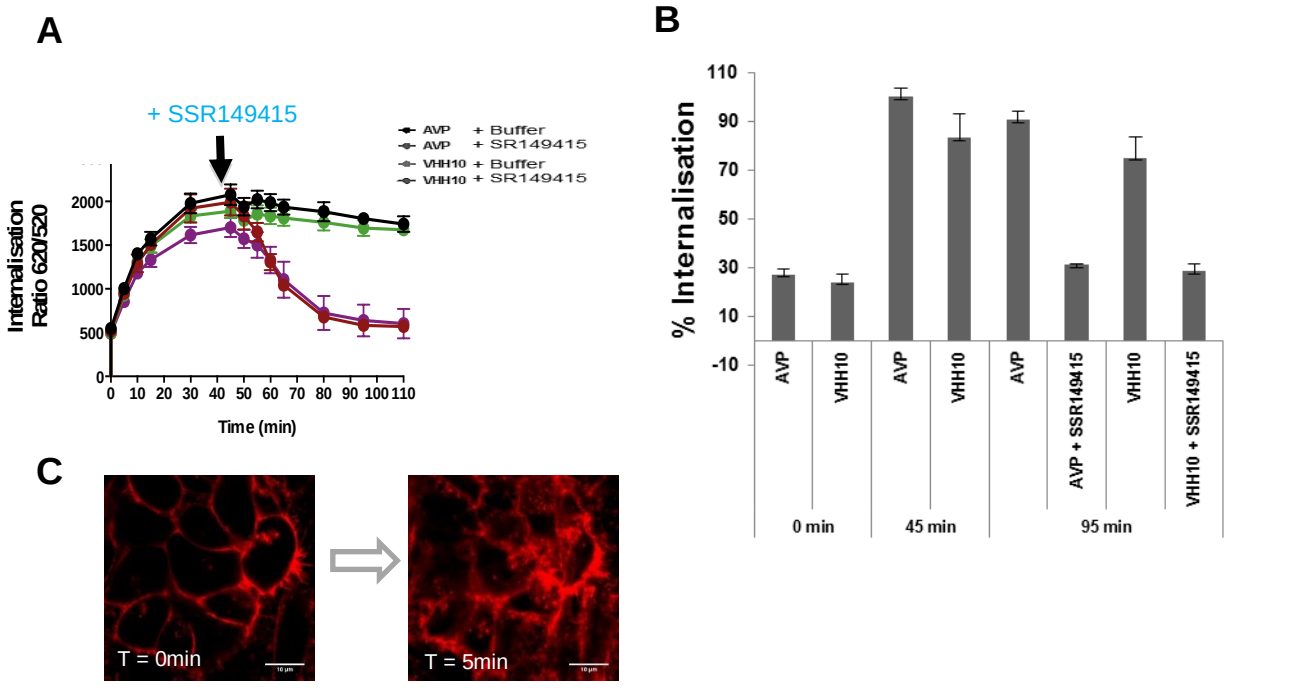


Figure 7: Detection of hV1B receptor induced internalization (*A) Real-time internalization and recycling of vasopressin V1B receptor. Following V1B internalization induced by AVP and VHH10 at 100 nM for 45 min, an excess of the antagonist SSR149415 (10 µM) was added (at the time (45 min) indicated by arrow) to stop the internalization, inducing the receptor recycling. Black and green lines represents V1B internalization induced by AVP and VHH10 (increase of the 620/520 ratio) respectively. Red (AVP) and purple (VHH10) lines represent V1B internalization and recycling illustrated by an increase and a decrease in 620/520 ratios, respectively. (B) Represent the percentage of internalization of V1B in presence of AVP and VHH10 at 100nM at T0 which represent basal level, T45 min the maximum internalization and recycling and at T95 min with and without addition of SSR149415. (C) Confocal microscopy of Hek TRex cells stably expressing hV1BR labeled with Strep-Tactin chromeo 546 before and after addition of VHH10 (100 nM). The internalization was then monitored at RT for 5 min. The data represent mean ± S.E.M from two independent experiments carried out in triplicates.*

Partner EPFL has resolved the spatiotemporal distribution of GPCRs and channel proteins using single molecule imaging and the newly EPFL developed procedure to determine mobility distributions of the receptor in the plasma membrane of living cells. Conventional confocal microscopy and STED microscopy has been used on model cells (HEK, CHO) and on cultivated primary cells. The studies in primary cells open the possibility for the first time to investigate GPCR spatiotemporal distribution in biologically/medically relevant cells. This opens the possibility to extend these kinds of measurements in the near future for determining simultaneously the position of two different proteins such as GPCRs and their G-proteins and to investigate how the local membrane concentration of interacting partners will influence the efficiency of trans-membrane cellular signalling reactions for which up until now no experimental data are available. Such data are of interest as input for modelling these networks and parameter estimation by software developed by NUID-UCD.

The following main results have been obtained

(i) 11 new Inducible stable cell lines have been established for the selected membrane receptors out of which 6 have been scaled-up and successfully purified.

(ii) Two intracellular targets have been expressed and purified.

(iii) All purified antigen proteins have been used for Llama immunization.

(iv) Three new antibody fragment libraries have been amplified and sub-cloned from blood-isolated PBMC of immunized llamas.

(v) Functional antibody fragments have been successfully selected on two pathway targets (Mek1 and ApocIII). New antibody fragments have been selected against Lpar2 and P2Y10 receptors; they still need to be characterised in functional assays.

(vi) A VHH activating the V1b receptor has been characterized.

(vii) Nanobodies are ideally suited to image single neuronal receptors in live cells. The differences of mobility patterns of the neuronal receptors between neuronal and heterologous cells show the importance to extend such investigations to bio-medical relevant primary cells. Furthermore, we have taken the first ultra-high resolution image of native receptors in neuronal cells.

4 Description of the potential impact

SynSignal was an international, intersectoral and innovative high-tech consortium that combined leading European research institutions and successful SMEs with excellent track records. The objective of SynSignal was to radically improve synthetic biology technologies and to create novel synthetic biology tools to accelerate progress in academic and industrial research in a wide range of applications. SynSignal actively supported the **Commission Europe 2020 Strategy** for smart, sustainable and inclusive growth, in particular the "**Innovation Union"** flagship initiative. Our work plan fostered a productive mindset, which fully supported **fluid exchange between the academic and private life science sectors**, and embraced the **entrepreneurial spirit**. SynSignal aimed to further the European Partnership for Researchers, across all intersectoral and international boundaries, to position Europe as a global leader in generating knowledge and added value in the 21st century. SynSignal particularly focused on **developing disruptive next generation high-throughput compatible synthetic biology research tools and technologies** for assembling synthetic signaling regulatory circuits, and to exploit them to recreate and modulate signaling pathways for health and disease, and for key industrial biotechnology applications. A paramount and integral objective of the work plan was to address **stringent quality control criteria** for all parts of the project. With its innovations, SynSignal contributed, **developed and validated new synthetic biology tools and technologies** that has and will enable the **generation of new knowledge**, with a prime focus on its translation into practical economic applications. SynSignal applied the innovative approaches it developed in its work plan for producing next generation products including **new therapeutics to interfere with signaling errors that cause human pathologies including cancer, and diabetes, novel delivery systems for modulating cell growth properties, custom engineered new cell lines for implantation, and next-generation screening platforms and next generation molecules with enhanced properties for the flavor, fragrance and health industries.** Notably, the work plan of our consortium answered the call to focus on new technologies that will **greatly accelerate discovery in the life sciences.** Our joint effort opened entirely new avenues for advancing large-scale European and global efforts that aim at catalyzing and exploiting the emerging field of synthetic biology, and industrial biotechnology. SynSignal tackled and overcame clear and present challenges and bottlenecks, which impede these fields in Europe and worldwide, to the benefit of industrial and academic R&D, and human health. SynSignal stood for **innovation**, catalyzed by drawing on the best available in European SMEs and the academic sector for its ambitious endeavor. SynSignal **developed, put-in-place, validated and exploited** next generation synthetic signaling technologies, next generation synthetic biology tools, and capitalized on these to provide new and better products.

In the **Europe 2020** strategy for growth, competitiveness in the future required first and foremost a "**strengthening of the sources of growth in Europe's industrial base**" (Barroso, 2009). The European pharmaceutical and biotech industries face increasingly difficult challenges from countries with significantly lower labor costs. To maintain on European soil highly paid, highly skilled jobs in these industries, **we must immediately develop competitive advantages that compensate for our higher labor costs**. The vision of SynSignal was to provide European pharmaceutical and industrial biotechnology companies with these competitive advantages through development of innovative technology platforms which change the way we discover and produce novel technology platforms, and novel products. **SynSignal's high throughput, synthetic biology-based technology platforms have and will boost both the speed and efficiency at which essential signaling pathways that dictate cellular processes can be modified, modulated and interfered with**, thereby reducing cost of product development, and speeding the time of delivery of new products to market. Due to the recent explosion of our understanding regarding the causes of disease at the genome, proteome and systems level, new approaches exploiting synthetic biology tools and technologies are bound

to dominate research and development towards new and potent drugs and therapies for the diseases that challenge the populations of Europe and strain our health care providers and pension plans with seriously disabled patients: **cancer, Alzheimer's and Parkinson's disease, auto immune and inflammatory diseases and others**. Our technologies will make accessible, for the first time, cellular signaling and metabolic pathways that are currently impossible to address using established technologies, thereby creating **opportunities to develop entirely novel classes of potent and efficient therapeutics**. The same platforms SynSignal created will also be simultaneously utilized to develop **new and better molecules for the fragrance, flavor and nutritional industries**, which are multibillion € markets. Strengthening of these markets with innovative technologies such as those SynSignal provided is imperative to strengthen and maintain Europe's leading position in this large and growing sector, thus maximizing the impact of research and innovation on European societies and economies. Synthetic Biology is a nascent, emerging technology with the potential to be transformational in a large number of key areas which address a highly diverse range of important socioeconomic challenges in healthcare, nutrition, green technology, and manufacturing. **The United States has led the way in synthetic biology as judged by publication output and investment.** In the last several years, the U.S. government has spent approximately $160 million on synthetic biology research per year, as compared to not even a fourth of that total in the leading European countries, outcompeting Europe in this viral and emerging field. This can be countered only with highest level technology development by the best European research teams and SMEs. **We formed the SynSignal team exactly with the objective to close this competitive gap.**

The results of the WP3 part of the project are a significant contribution to improve and speed up the future research in the field of taste, both in basic research and in industrial applications. The project has brought new findings and tools which will be helpful in screening processes for new taste-modifying chemicals, aimed at identifying substances with a potential to increase the acceptability of healthy food (such as low sugar or low fat products) or aversively tasting medicines. Thus, they will contribute to the global efforts to improve health in our society and combat conditions such as diabetes or obesity. The knowledge gained in the course of the project will also influence and direct future research investigating the mechanisms underlying taste perception, and the role of taste receptors in tissues outside the oral cavity. A better understanding of those processes will eventually make it possible to tap the potential of taste receptors as drug targets.

WP4 delivered a number of GPCR activating compounds which are of interest for fragrance development and therapeutic applications.

WP7 resulted in the selection and characterization of the first GRCR targeting VHH with a full agonist profile. The extremely high selectivity of this type of ligand open the door to numerous therapeutic potentialities considering the tremendous importance of this class of target in the actual and future drug discovery area.

5 Address of project public website and relevant contact details

Website: synsignal.eu

Contact: Prof Horst Vogel
École Polytechnique Fédérale de Lausanne
+41 216933155
+41 216936190
horst.vogel@epfl.ch

The SynSignal Consortium

École Polytechnique Fédérale de Lausanne

University College Dublin, National University of Ireland

Karolinska Institutet

Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke

Fundació Privada Centre de Regulació Genòmica

Bio-Xtal Sárl

GBiotech Sárl

European Research and Project Office GmbH

University of Bristol