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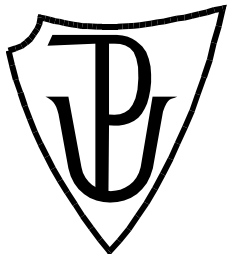

Generation and characterization of stable expressing cell lines using different approaches for targeted integration into genome of mammalian cells.

Master thesis

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„I declare that this submitted thesis was worked out individually using referenced literature“.

In Olomouc, …… ……

I would like to express my appreciation to whole Amgen Company for a great chance and embedded confidence. Thanks for giving me the opportunity to be part of the Amgen research. Special thanks belong to my supervisor Markus Hierl, Dipl.-Ing. for his time, patience and understanding, it has been an honor to work with you. Also thanks to Dr. Ulrike Schindler for making this study possible, Dr. Ralf Schwandner and Dr. Holger Beckmann for their advices, help and wonderful supervision. My special thanks belong to Mr. Mirko Stemmler for his help with all office staff and his optimism, Mrs. Gerda Lang and Mrs. Ulrike Schnell for perfect technical services and to all my colleagues from ARG Team: Simmone Strauch, Katja Labitzke, Sabine Tyrra, Tanja Fisch, Franziska Drückenmüller, Marion Nelles, Michael Henkel, Niels-Erik Enkler, Johannes Knop, Peter Jäckel, Kerstin Hasslinger, Silvia Materna-Reichelt and Anke Konrad.

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Jméno a příjmení autora	Bc. Petra Koudelková
Název práce	Příprava a charakterizace stabilně exprimujících buněčných linií s využitím různých přístupů pro cílenou integraci do genomu savčích buněk.
Typ práce	Diplomová
Pracoviště	Amgen Research GmbH, Regensburg, Německo
Vedoucí práce	Markus Hierl, Dipl.-Ing.
Rok obhajoby práce	2012
Abstrakt	Receptory asociované s G-proteiny (GPCRs) představují širokou skupiny membránových proteinů. Tyto receptory jsou v současnoti nejvíce zkoumanými potenciálními cíli ve farmaceutickém průmyslu. Díky jejich schopnosti odpovídat na široků spektrum vnějších signálů a všudypřítomné expresi v organismu představují GPCRs široké možnosti mezi terapeutickými cíli. Receptor asociovaný s G-proteinem 34 (GPR34) patří mezi skupinu GPCRs označovanou jako tzv. “osiřelé receptory”, protože u nich dosud nebyla zjištěna jejich fysiologická funkce ani přirozený agonista. Tento receptor jsme si vybrali jako náš gene zájmů pro porovnání tří různých strategií pro integraci do genomu a tvorbu stabilních linií. Také jsme tento receptor otestovali v high-throughput screeningovém testu, aby tento receptor nemusel být i nadále sirotek.
Klíčová slova	hGPR34, G-protein, GPCR, receptor asociovaný s G-proteinem, Jump-In, Gateway, Meganuklease, cílená integrace, stabilní buněčné linie, high-througput screening
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Abstract	G-protein coupled receptors (GPCRs) represent a wide class of integral membrane proteins. These receptors are currently the most investigated drug targets in the pharmaceutical industry. Due to their response to miscellaneous external stimuli and ubiquitous expression in organism GPCRs represent a wide range of opportunities among therapeutic targets. G-protein coupled receptor 34 (GPR34) belongs to the GPCR group called "orphan receptors" since we do not know any agonist and physiological function of this receptor. We picked this receptor as our gene of interest for comparison of three differet strategies for integration into genome and generation of stable cell lines. Future plans are to try to test this receptor in a high-throughput screening to find a surrogate ligand and further investigate the physiological role of this GPCR.
Keywords	hGPR34, G-protein, GPCR, G-protein coupled receptor, Jump-In, Gateway, Meganuclease, targeted integration, stable expression, high-throughput screening
Number of pages	86
Number of appendices	0
Language	English

Purpose of thesis

- Summarize the actual knowledge about GPR34 and different cloning approaches
- Construction of a mammalian cell line stably-expressing human GPR34
- Characterization and functional testing of the stable clones
- Comparison of different methods for integration into the genome.

Contents

1. Theoretical part

1.1 Introduction

Since the decoding of the human genome in 2003 has passed only a few years ago, the first idea of DNA retargeting is much older. The first thought about the „replacement of faulty genes in several genetic diseases with the good working copies“, dates back to the 1960´s (Friedmann, 1992).

The first clinical trial on this topic was performed by Dr. Stanfield Rogers in 1970 (Sheridan, 2011). Patients were two German sisters suffering from argininemia, a genetic disorder where the body does not produce an enzyme called arginase (the gene encoding sequence for arginiase is localized on chromosome 6), which catalyzes the conversion of arginine to ornithine and urea. When the body lacks this enzyme, it leads to the accumulation of arginine and urea in the blood. As urea is the molecule carrying most of the wasted body nitrogen, its abundance causes (apart from other symptoms) cerebral atrophy and mental retardation (Saheki et al., 1987). In earlier studies with rabbits, Dr. Rogers observed lower levels of arginine in the animals after infection of papillomavirus. He treated both sisters with the Shope Papilloma Virus (SPV) believing, that the presence of the viral DNA could „repair” the expression of the default gene. His trial did not bring any benefit to the patients, but it can be considered to be the first milestone in the history of the gene therapy and DNA retargeting.

Let us focus closer on this experiment: due to the knowledge we have today about genetic engineering, we can observe two important facts: the first one is the use of the virus as the vector particle. This fact is very important and different viruses are being used for infection (transfections of cells where a virus is the vector) until nowadays. The second fact is the absence of the appropriate DNA molecule. Lower arginine level in the experiment with rabbits is probably caused by viral infection and not by insertion of the new gene for arginase. As we know today, for a successful change in expression profile, the presence of engineered DNA sequence in the carrier particle is necessary. We cannot blame Dr. Rogers from negligence of any kind, because the knowledge about restriction endonucleases was still at its beginning (*Hind*III was described as the first one in 1970, other endonucleases followed during the 1970´s) (Smith & Wilcox, 1970).

Since the 1970´s many different techniques of DNA delivery into cells have been developed, and all are facing one particular problem. Once the DNA is inside the cell, it has to overcome all the degradation processes and needs to be inserted in the genome to ensure stable expression over time. When we consider the enormous size of the

human genome and also the fact that only about 2% of it are coding sequences that can be translated, we have to state that a stable integration into genome is primarily a coincidence.

1.2 G-Protein coupled receptor 34 (GPR34)

„If you had to make a wild guess about the target of a certain drug, your best odds are with G-protein coupled receptor." (David Filmore)

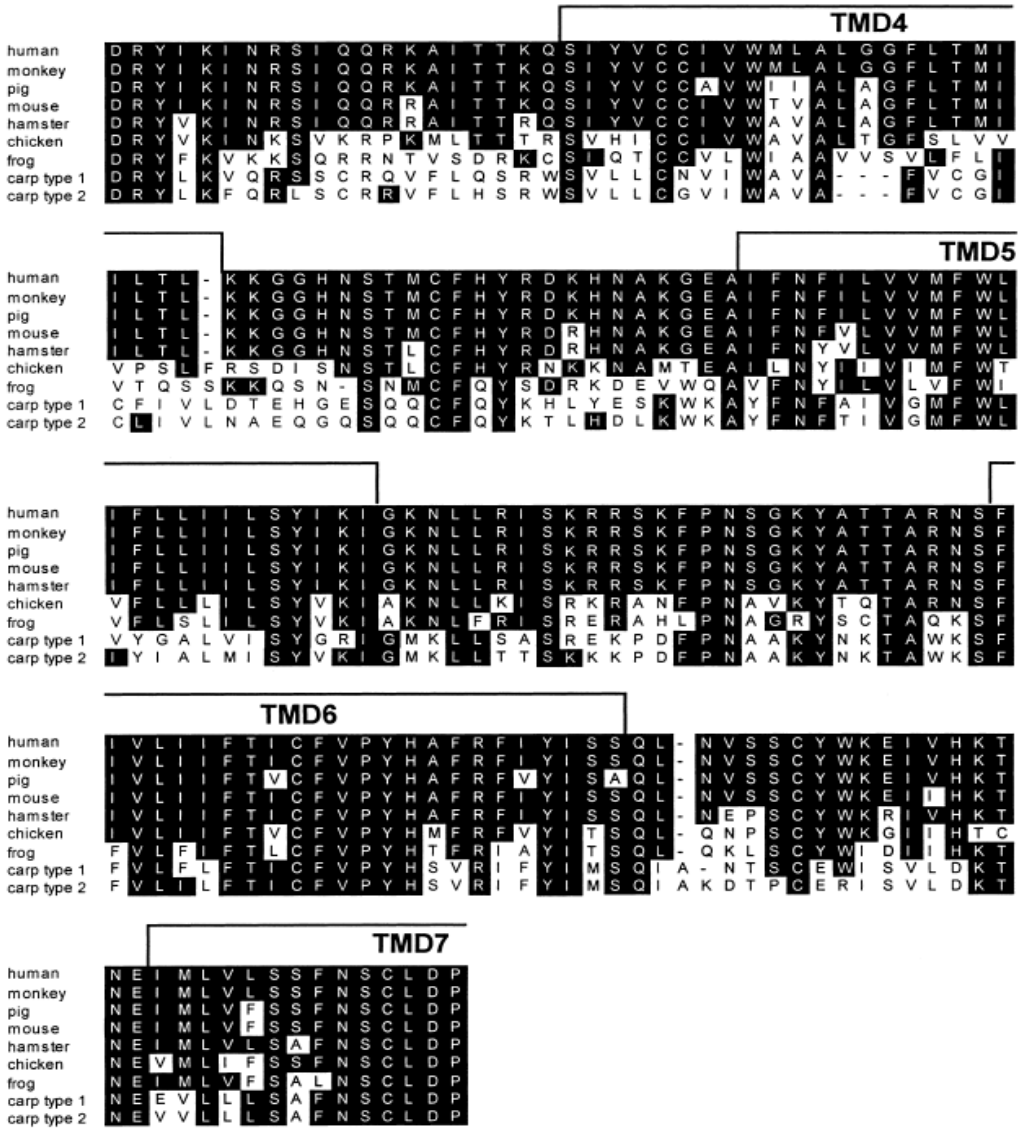
GPR34 was discovered in 1998 by human EST (Expressed Sequence Tags) analysis (Marchese et al., 1999) and described as a translational ORF (Open Reading Frame) of 1143 bp (base pairs) encoding a 381-amino acid protein. Analysis of the human genomic DNA revealed that the new gene contains all structural features of the class I of G-Protein Coupled Receptor Family (Schöneberg et al., 1999). No endogenous ligand has been identified as of yet, so the new receptor has been classified as an „orphan receptor" (GPCR receptors with no known ligand) and considering orphan nomenclature it has been named GPR34 (Marchese et al., 1999), (Schöneberg et al., 1999).

Further studies localized GPR34 on X-chromosome p11.3 - 11.4 (Schöneberg et al., 1999). In addition, GPR34 is present as an intronless single-copy gene and its sequence seems to be highly conserved among vertebrates. For example, the identity between human and mouse cDNA is 86% at the nucleotide- and 90% at the amino acid level (Schöneberg et al., 1999).

Due to the localization on the X-chromosome, questions of any connection between GPR34 and some hereditary genetic diseases did arise. One example is congenital stationary night blindness (CSNB), a non-progressive genetic disorder predominantly effecting males (Online Medical Dictionary, www.medterms.com). Two distinct loci for CSNB are localized on short arm of chromosome X. CSNB type 1 also called „complete" disorder is conditioned by mutations in NYX gene (Nyctalopin) (Boycott et al., 1998). In CSNB type 2 the phenotype for the disease is incomplete. Here the gene CACNA1F, encoding a calcium voltage-gated ion channel is affected. The predominant phenotype for CSNB1 mutations is worse sight at dark (rods are impaired), for the CSNB2 it is a slower response to changes in light conditions (Lodha et al., 2010), (Boycott et al., 1998). Screening of cDNA from Xp11.3 – 11.4 specified the position of GPR34 to the Xp11.4 region, thus it did not confirm any involvement in the congenital stationary night blindness (Jacobi et al., 2000). Breakpoints and micro deletion at the Xp11.4 are found as well in Turner syndrome and mucosa-associated lymphoid tissue lymphoma but lack of GPR34 is not a primarly cause of these disease states (Liebscher et al., 2011).

1.3 GPR34 – Basic structure and characterization

As previously mentioned, GPR34 is present in the genome as an intronless single-copy gene on X-chromosome p11.4. The receptor seems to be very old as layed foundations of this receptor can be found in cartilaginous and ray-finned fish genomes (Schulz & Schöneberg, 2003). This indicates that the evolution of GPR34 likely started more than 450 million years ago. Therefore is it more than interesting that GPR34 demonstrates a very high evolutionary conservation among vertebrates („Pic. 1", Schöneberg et al., 1999).

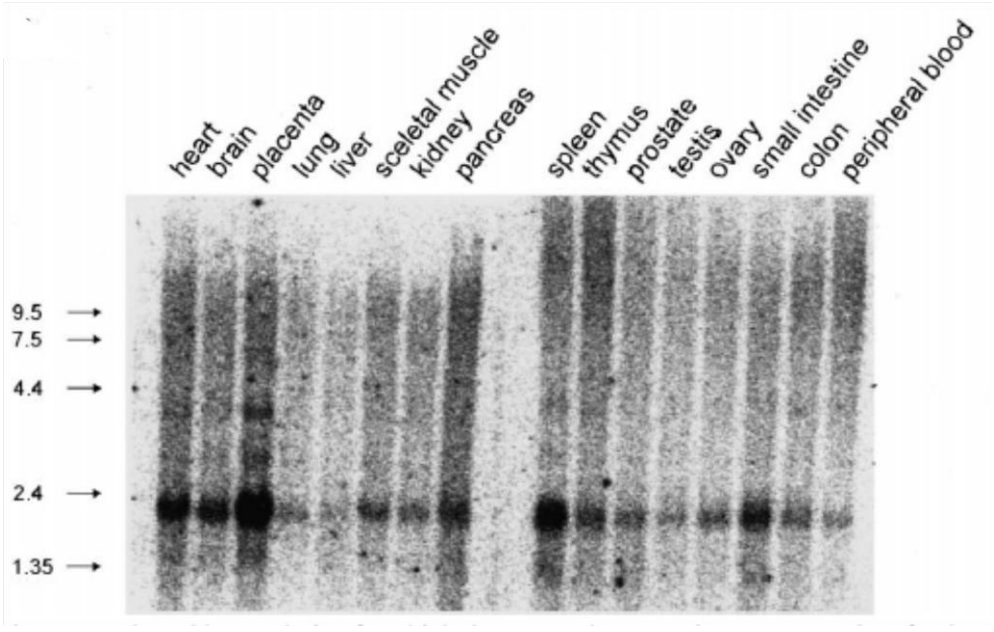


„Pic. 1": GPR34 orthologs and their amino acids comparison between human (*Homo sapiens*), monkey (*Cercopithecus aethiops*), pig (*Sus scrofa domesticus*), mouse (*Mus musculus*), hamster (*Cricetulus criseus*), chicken (*Gallus gallus domesticus*), frog (*Xenopus laevis*) and carp (*Cyprinus carpio*). PCR with degenerated primers for a region of TM3 to TM7 was performed and sequences of samples were analysed. From carp genomic DNA were obtained

two different GPR34 clones. Amino acid comparison reveals high evolutionary conservation among vertebrates (Schöneberg et al., 1999).

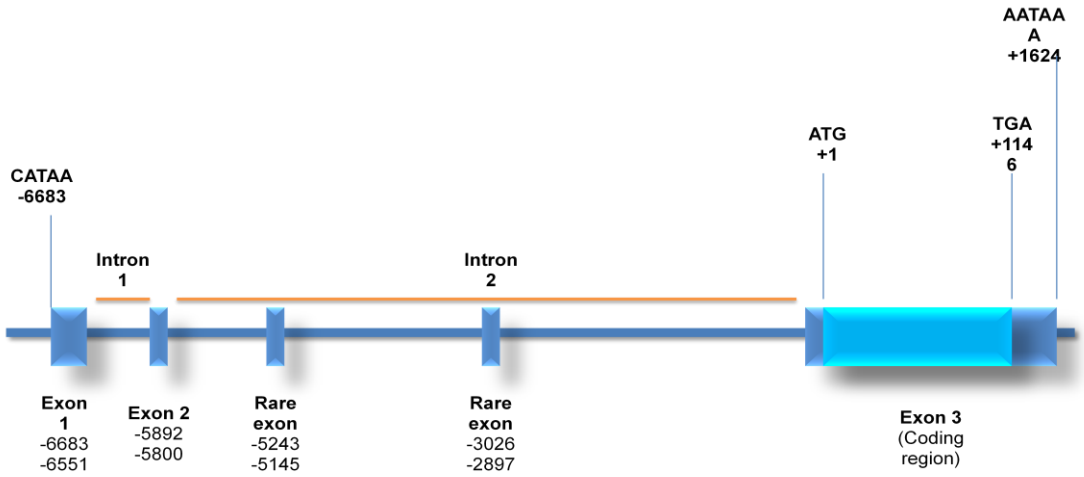
In an analysis focused on allelic diversity among humans, 20 individuals of Caucasian origin were investigated (Engemaier et al., 2006). Except of one male hemozygous with silent mutation at codon position 296 GTA/GTG, there were no other sequence variations found. Due to a relation of genetic diversity with linguistic groups, 85 members from all major linguistic groups were inspected. However, sequence variations within the coding region or single nucleotide polymorphisms were not found (Engemaier et al., 2006).

The question was asked if there are actually any possible variations in the structure of GPR34. On the level of mRNA it was found by Northern blot analysis that 1 band of approximately 2 kb in human and 2 bands of the length of 2,1 kb and 2,3 kb respectively in mouse are present in all investigated tissue samples („Pic. 2", (Schöneberg et al., 1999). Subsequent experiments in mice revealed presence of two different promoters (Engemaier et al., 2006). One so-called proximal promoter is equivalent to a promoter also found in human. The second one, called distal promoter is located 700 bp upstream of the main transcriptional start. Comparison of both transcripted products confirmed an existence of both mRNA fragments. Moreover both promoters seem to be active in all investigated murine tissues (Engemaier et al., 2006).



„Pic. 2": Northern blot analysis of human tissue samples. hGPR34 transcripts were detected by Multiple Tissues Northern blots (Clontech) with P^{32} – labeled DNA fragments covering the complete hGPR34 ORF (Schöneberg et al., 1999)

Further sequence comparison revealed a conserved 5´exon-intron structure within the 5´UTR (Five prime untranslated region) (Engemaier et al., 2006). 5´UTR is a region prior to a gene between the transcriptional start and the start codon. 5´UTR represents an important tool for gene expression control because its sequence may influence mRNA stability and translational efficacy. Although these sequences are not translated, they may provide a binding sites for regulatory proteins and RNA, some of them may function as IRES (Internal Ribosome Entry Site – sequences which enable a ribosome to bind and translate mRNA not only from the 5´cap beginning but also from the middle of mRNA) or influence the translation by the presence of upstream ORFs (Hughes, 2006). Sequence of the 5´UTR may vary from cell to cell due to tissue membership and its uniqueness seems to be an important factor determining the destiny of future protein. Exons and introns quantity within 5´UTR can contribute as well to unlike protein-variants due to alternative splicing. In the case of GPR34 at least two additional exons within the 5´UTR region were discovered to be present in almost all investigated cDNA library transcripts, plus two additional so-called rare exons although with lower frequency („Pic. 3"), (Engemaier et al., 2006).



„Pic. 3": Brief scheme of the structure of human GPR34 transcripted region. Transcription start occurs within exon 1 approximately 6,5 kb upstream the coding region. (Self-made picture, based on Engemaier et al., 2006).

It was discovered during sequence analysis, that up to 5 in-frame AUGs are located within the first 150 bp's of the region, corresponding to the N-terminal domain of the translated protein. An experiment was designed to reveal which one of these AUGs is actually responsible for the translation (Engemaier et al., 2006). The first three in-frame AUGs were mutated to TTG in GPR34-GFP fusion constructs. Surprisingly all of these AUGs can initiate massive translation (Engemaier et al., 2006). The presence of

several alternative AUGs may give rise to different receptor isoforms, as it has been demonstrated for other proteins (Liu et al., 2000), (Porras et al, 2006).

1.4 G-Protein coupled receptors

G-Protein Coupled Receptors (GPCRs) represent a wide family of membrane integrated receptors. In mammalian cells, they are one major regulator of cellular response to different external signals which can be photons, neurotransmitters, nucleotides, phospholipids, proteins, hormones, peptides etc. Since completion of the sequencing of the human genome, more than 900 unique G-proteins have been identified. Among those, two thirds comprise olfactory and light receptors (Liebscher et al., 2011). GPCRs share a common structure: the contain of an extracellular N – terminus, seven hydrophobic transmembrane α-helices (7TM) and a cytoplasmatic C – terminus with 3 exo- and 3 cytoloops (Ji et al., 1998).

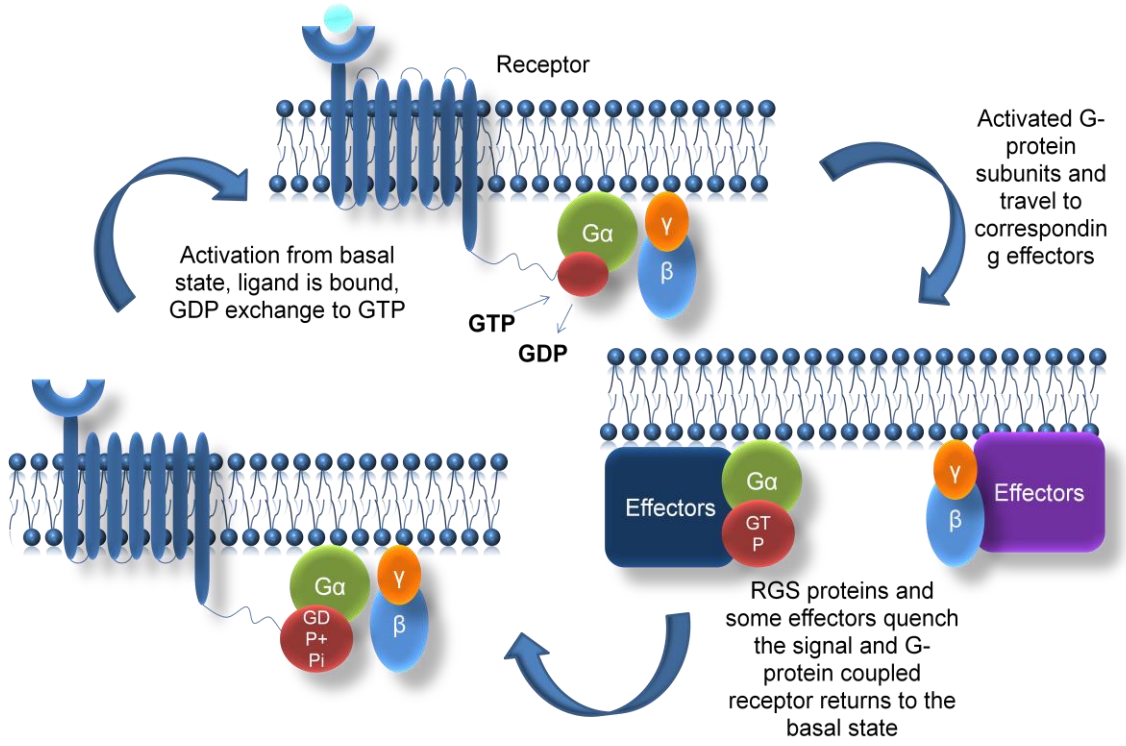
All the family can be divided into many groups and subgroups according to similarities in sequence homology, ligand structure and function (Ji et al., 1998). GPR34 belongs to the class I GPCR family (Rhodopsin-like) $P2Y_{12}$-like receptor group. This group includes ADP receptors $P2Y_{12}$ and $P2Y_{13}$, UDP-glucose receptor $P2Y_{14}$ and the orphan receptors GPR87, GPR82 and GPR34 (Marchese et al., 1999).

As it follows from the name of GPCRs, these receptors do not operate alone. There signaling is coupled to so-called G-proteins, heterotrimeric enzymes with GTPase activity associated to the cytoplasmatic membrane. G-proteins are built from 3 different subunits α, β, γ. Both subunits α and γ are covalently attached to a membrane, and subunits β and γ together forming a single functional unit. Subunit α contains GTP/GDP-binding site and can be reassociated from the protein complex and subsequently activate different signaling cascades. It is traditionally the Gα subunit which awards a specific function to the respective G-protein. However Gβγ-mediated signaling and interaction with effector molecules are also described in the literature (Clapham & Neer, 1997).

Coupling and uncoupling of receptors to effector molecules is cyclic. In the inactive state, the αβγ-subunits form a heterotrimer with GDP bound to the Gα-subunit binding site. When a signal from receptor is received, GTP binds instead of GDP and the Gα-subunit is allowed to move freely from the trimer as well as the Gβγ-complex to promote and modulate effectors like enzymes engaged in signaling cascades or different ion channels. Inactivation is conditioned by hydrolysis of GTP to GDP due to

α-subunit GTPase activity, which leads to reassociation into the trimeric complex („Pic. 4"), (Smrcka et al., 2008).

It is more than obvious that the deciding step for reassociation kinetics is GTP hydrolysis. Surprisingly, isolated G-proteins show lower GTPase activity compared to those under physiological conditions. An idea of supporting mechanism enabling modulation has been postulated and several so-called RGS proteins (Regulators of G-protein signaling) were later discovered. Moreover these RGS may influence the specificity of G-protein signaling or have itself effector functions („Pic. 4"), (Wettschureck & Offermanns, 2005).



„Pic. 4": Simplified scheme of G-protein action. In the basal state when no ligand is bound, forms G-protein a trimer with GDP present in Gα binding site. After agonist binding GDP is being exchanged for GTP and the Gα-subunit and/or Gβγ complex travel up to effector molecules. Inactivation and return to the basal state is accompanied with GTP/GDP exchange with the support of RGS. (Self-made picture based on Wettschureck & Offermanns, 2005).

G-proteins are divided into different groups with respect to the varieties in the α-subunit: G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$.are some examples. These groups are based on the sequence homology between members and further arranged into subgroups according to their target in cells (Hurowitz et al., 2000). The G-protein βγ-complex was traditionally referred to as a passive partner to the „more occupied" Gα-subunit,

nevertheless it was confirmed that variability does exist among βγ-subunits and that the importance of the βγ-complex cannot be ignored (Clapham & Neer, 1997). Moreover there are several cases where ligands that bind to the receptor lead to the activation of both Gα-subunit and βγ-complex. This is very common when the α-subunit is $G_{i/o}$. Diversity between βγ-complex signaling is enhanced by the existence of 5 different types of β-subunits and 12 γ-subunits (Wettschureck & Offermanns, 2005).

1.5 Signaling cascades and main targets of G-proteins

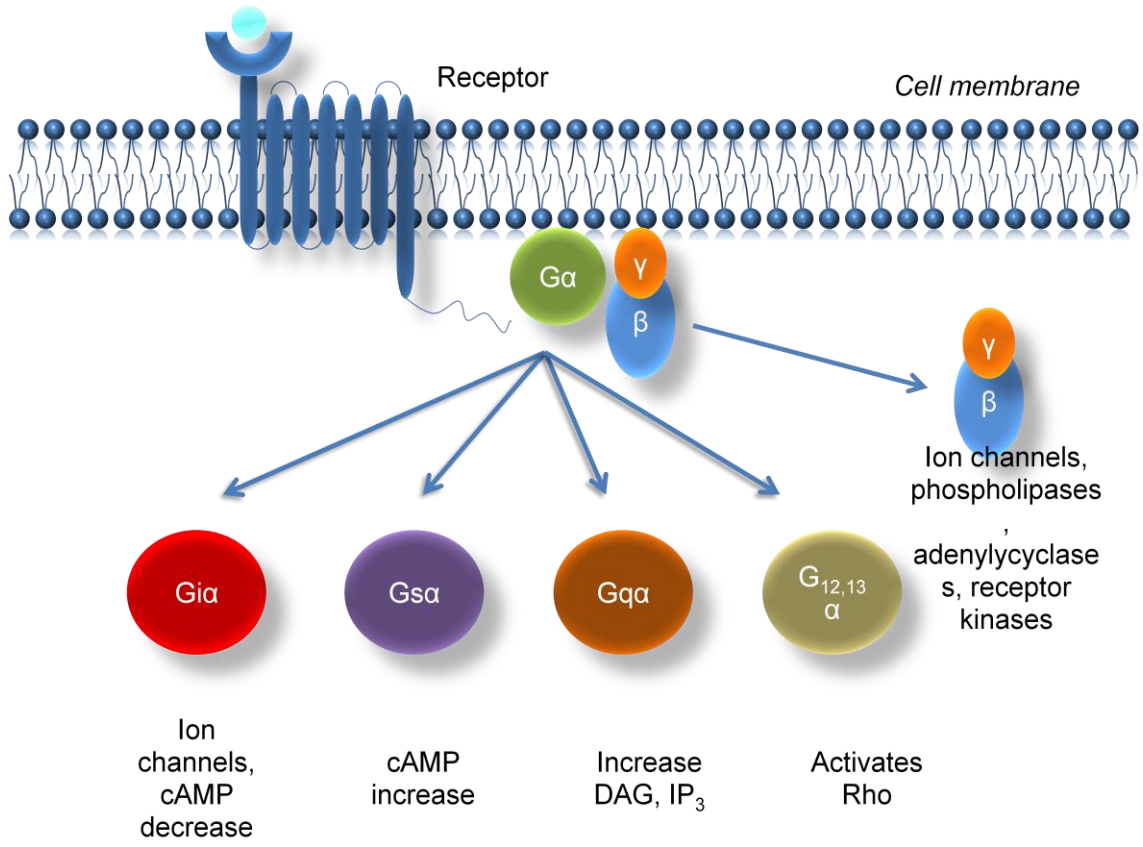
Independently on their coupling to different receptors, G-proteins share targets and signaling cascades. Most important targets and subsequent pathways which may be influenced via G-proteins are those involving adenylyl cyclase and phospholipase C. G-proteins have also an ability to act trough activation of ion channels (Chen-Izu et al., 2000). Most receptors are not coupled to only one G-protein. Their activation may lead to launch of several signaling cascades which means the cellular response is more complex than is seems (Wettschureck & Offermanns, 2005).

Adenylyl cyclase is a membrane bound enzyme which catalyzes a conversion of ATP (adenosine triphosphate) to cAMP (cyclic adenosine monophosphate) – an important small second messenger molecule within cells. cAMP activates PKA (cyclic-AMP-dependent protein kinase or protein kinase A) by removal of a regulatory subunit from the inactive protein and allows the enzyme to propagate signals. cAMP also directly open calcium ion channels or act indirectly as a factor regulating transcription for several genes which contain a so-called CRE- (cyclic-AMP-response element) domain through PKA activated CRE-binding proteins (Alberts et al., 2008).

There are at least 9 isoforms (I – IX) of adenylyl cyclase (Hanoune & Defer, 2001) which are almost exclusively bound to Gs-receptors („Pic. 5"), (Billington & Penn, 2003). Due to the rise of the levels of cAMP during Gs-activation (from 10^{-7} M to more than 20-fold), the cAMP level is often used during G-protein coupled receptor investigation in *in vitro* assays and also in HTS (High Throughput Scrrening) tests e.g. AlphaScreen® (PerkinElmer) or cAMP HTRF® technology (Cisbio).

The principal effector of Gq-coupled receptors is a membrane-bound enzyme called phospholipase C (PLC), which hydrolyzes phosphoinositol 4,5-bisphosphate (PIP_2) into two second messenger molecules – 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) („Pic. 5"). The existence of 11 different PLC isoforms has been

confirmed and they are further divided into several subfamilies (Rhee, 2001). Gq-coupled receptors are predominantly linked to PLC-β subfamily.



„Pic. 5": General distribution of G-protein influenced targets based on the type of activated subunit (Self-made picture based on „Diversity of G Protein-Coupled Receptor Signal Transduction Pathways", Sigma Aldrich).

Propagation of the Gq-mediated signal through IP_3 is aimed to towards the endoplasmic reticulum (ER) where it binds and opens so-called IP_3-gated Ca^{2+} ion channels. Ca^{2+} release from ER lumen is very fast due to the concentration gradient and Ca^{2+} concentration in cytoplasm may rise very fast from 10^{-7} M in resting cell to more than 10-20 – fold in activated cells. Ca^{2+} serves as another second messenger and once released it may affect other calcium-dependent proteins e.g. protein kinase C (PKC) or calmodulin.

Hydrophobic DAG, the second product of the PIP_2 hydrolysis, remains bound to the inner side of the cytoplasmatic membrane where it has two main functions. First it helps to activate PKC in combination with Ca^{2+} released by IP_3. Second it can be further cleaved to produce arachidonic acid which may act as a signal molecule or be

used for the synthesis of eicosanoids. The changes in intracellular Ca^{2+} levels are also historically utilized in *in vitro* assays, e.g. Aequorin-based assays, similarly to cAMP.

Gi/o-coupled receptors seem to be the most abundantly expressed subgroup of G-proteins in most cell types (Billington & Penn, 2003). Activation of Gi/o-coupled receptors leads to inhibition of Gs-stimulated adenylyl cyclase thus lowering cAMP concentration in cell. Gi/o-coupled receptors are also believed to be the largest source of βγ-mediated signaling due to the high expression pattern through many different cells („Pic. 5"). GPR34 is supposed to be a Gi/o-coupled receptor (Sugo et al., 2006).

The G12/13 group of G-protein coupled receptors is not as good characterized as the other groups. Associated receptors as well as the corresponding signal pathways were not yet established with the exception of guanine nucleotide exchange factors for the small G-protein Rho („Pic. 5"), (Sah et al., 2000).

1.6 Biology and physiology of GPR34

GPR34 exhibits an ubiquitous expression pattern through different tissues and among species (Schöneberg et al., 1999). Moreover, its expression seems to be constitutively active (Engemaier et al., 2006).

Sugo et al. postulate an increased GPR34 expression primarly in mast cells – granules containing cells that release a wide range of biological mediators such as histamine, leukotrienes, prostaglandins, proteases and cytokines into the interstitium (Sugo et al., 2006). Mast cells are mainly known for their role during inflammatory response when among other effects antigen stimulation results in the histamine release. This reaction is greatly supported in the presence of lysophosphatidyl-L-serine (lyso-PS) (Martin & Lagunoff, 1979). Lyso-PS is the product of hydrolysis of membrane lipids through phospholipases A1 and A2 after phosphatidylserine exposition to cell surface during apoptosis (Aoki et al., 2002). Due to high GPR34 expression in mast cells and lyso-PS mediated degranulation of mast cells, the idea has been raised that lyso-PS could be the desired agonist for GPR34 (Sugo et al., 2006).

Phylogenetic relationships between GPR34 and other receptors like UDP-glucose receptor and P2Y nucleotide receptor family suggest that GPR34 could also be one of the possible receptors for low molecular weight molecules such as nucleotides, nucleotide sugars and phospholipids (Sugo et al., 2006). CHO (**C**hinese **H**amster **O**vary) cells stably transfected with hGPR34 cDNA were tested to a variety of tissue extracts after HPLC fractionation (Sugo et al., 2006). For this, samples were taken from

rat and porcine brain tissue. One of these fractions evinced an inhibitory effect to forskolin-induced cAMP accumulation (ELISA kit, AmershamBiosciences). Forskolin (coleonol) is often used to resensitize G-protein coupled receptors by direct activation of adenylyl cyclase. Comparison between retention times of the active fraction and compound libraries revealed that the part of the active fraction has corresponding time to those known for forskolin and thus it could be the searched molecule. An experiment with RPMCs (Rat Peritoneal Mast Cells) revealed an enhanced degranulation after 100 nM lyso-PS stimulation. These findings led to conclusion that lyso-PS is the desired GPR34 agonist (Sugo et al., 2006).

To investigate the physiological importance, GPR34-deficient mice were generated. These mice did not show any anatomical or histological abnormalities and changes in behavior (Liebscher et al., 2011).

The same group verified if the lyso-PS really is a GPR34 agonist. Transiently transfected as well as stable mouse and human GPR34 expressing COS-7 cells were pre stimulated with 10 µM forskolin and tested for cAMP level changing using Alpha Screen® Technology. This experiment was supported by a Fura-2 calcium measurement assay. Unfortunately no reductions in cAMP and Ca^{2+} levels were observed.

To find out if there is not a connection between GPR34 and phospholipase C/inositol phosphate pathway, an inositol phosphate accumulation assay was performed. Similarly, no specific response upon lyso-PS stimulation was observed (Liebscher et al., 2011).

As previously mentioned, GPR34-deficient mice did not show any apparent abnormalities. Additionally no other changes in body arrangement and development, weight of organs or serum parameters were observed. In behavioral assays GPR34-deficient mice showed reduced activity in the OFT (**O**pen **F**ield **T**est – an experiment to test rodents emotionality), but increased activity in Light-dark test (an experiment based on the natural tendency of rodents to prefer dark environment) when compared to the wild type mice (Liebscher et al., 2011).

So the relevant question is what actually happens when we knock out the GPR34 gene? Is this receptor really vitally important as it is so highly evolutionary conserved or we can easily live without it?

To answer this question we should first look at information about the GPR34s expression pattern. Sugo et al. postulated increased GPR34 expression in mast cells.

This statement can be made for all mononuclear cells of the immune system (Liebscher et al., 2011). Previously mentioned GPR34-deficient mice in comparison with wild types were immunized with MBSA (Methylated Bovine Serum Albumin) and deficient mice showed significantly lower number of granulocytes and macrophages in spleen (Liebscher et al., 2011).

Microglia cells, a specific type of macrophages derived from hematopoetic stem cells during hematopoiesis, are an important part of the immune system. Firstly, they have to differentiate from the stem cells in bone marrow into monocytes and further travel to the CNS (Central Nervous System) where they differentiate into microglia. As most of the antibodies are unable to cross the brain-blood barrier due to their size, microglia take over this task and cater for basic immunity of the CNS. Once activated by stimuli, microglia proliferate vigorously, release cytotoxic and inflammatory mediators,and strongly express both classes of MHC (Major Histocompatibility Complex) – MHC I as well as MHC II antigens and transform into phagocytic cells (Gehrmann et al., 1995).

Even if the cells of the immune system share the same origin, once differentiated, they show fundamental differences. In a study where microglia, monocytes and spleen macrophages were investigated and their transcriptional profiles were compared, it was found that microglial cells exhibit high expression of the GPR34 (Bédard et al., 2007). Moreover, GPR34 gene was strongly upregulated when microglia treated with cuprizone, a demyelinating agent, suggesting GPR34 may play a role during neuroinflammation (Matsushima & Morell, 2001).

This statement is in a good agreement with an experiment performed by Liebscher et al. GPR34-deficient mice were infected with *Cryptococcus neoformans*, an encapsulated yeast-like fungus which can affect both plants and animals. After pathogen inhalation, an infection causes predominantly systemic cryptococcosis, but for example in patients suffering from AIDS this can cause fungal meningitis - an inflammation of the protective membranes covering brain and spinal cord. Although the survival rate after pathogen exposition did not show any significant difference between WT and KO (knocked-out) mice, GPR34-decifient mice showed higher pathogen burden especially in lung, spleen and brain (Liebscher et al., 2011). Interestingly GPR34-KO mice showed increased basal levels of several cytokines, especially TNF-α (Tumor necrosis factor-alpha), GM-CSF (Granulocyte-macrophage colony-stimulating factor), and IFN-γ (Interferon gamma) when compared to the WT. Although cytokine levels are increased in WT mice after re-exposition to the pathogen, their value did not even reach basal cytokine levels found by non post-infected KO individuals (Liebscher

et al., 2011). In conclusion, higher cytokine levels, higher pathogen burden and lower efficiency in the defense against *Cryptococcus neoformans* might be caused by an inadequate activation of the immune system of the GPR34-deficient individuals (Liebscher et al., 2011).

These findings indicate that G-protein coupled receptor 34 may be involved in inflammatory processes/diseases but still need more proof. Firstly we need to find out the natural agonist through cell based assays and determine associated signaling cascades. Subsequently we have to find physiological importance and the pharmacological role of this receptor.

1.7 Cell based assays used in GPCR pharmacology research

High throughput assay formats allows the pharmaceutical industry to test many compounds in parallel and faster rather than common laboratory methods. Due to the GPCRs existence between external and internal space, leader among other approaches are cell-based screening assays. One of the keys is to prepare cell lines with confirmed and desired levels of receptor expression. One very popular immortalized cell line are CHO (**C**hinese **H**amster **O**vary) cells because of their high protein production and fast growth or COS (**C**V-1 simian in **O**rigin and carrying **S**V40) cells.

Once the cell lines are established, they can be use in different assays. Most GPCR HTS assays are based on the measurement of intracellular second messenger levels – Ca^{2+}, cAMP or IP3. In the next paragraphs I will briefly describe several assays like aequorin-based assays, calcium-based assays, two cAMP assays (cAMP AlphaScreen® assay and cAMP HTRF® assay and quite new and versatile Tango® GPCR assay format.

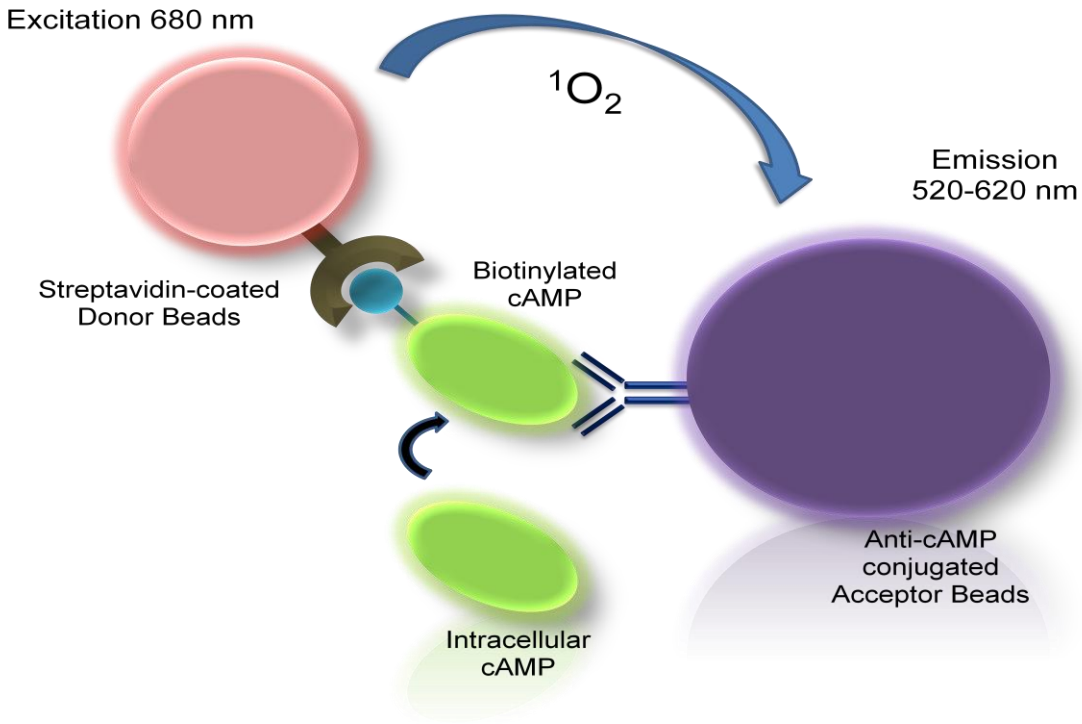
Aequorin-based functional assays are used to monitor Ca^{2+} levels in the inner environment of cells. They are based on an interaction of Ca^{2+} ions with the photoprotein aequorin originally isolated from luminescent jellyfish (*Aequorea Victoria*) (Shimomura et al., 1962). The Aequorin protein is composed out of the apoprotein apoaequorin and the prosthetic group coelenterazine – luciferin, responsible for light emitting. The functional protein contains three EF hands (helix-loop-helix binding domains) enabling to bind calcium ions. Once calcium ions are bound, the protein changes its conformation and the prosthetic group undergoes an oxidation which converts coelenterazine into excited coelenteramide. Relaxation and return to the

ground state is accompanied with a light emission of the wavelength 469 nm. Apoaequorin can be easily expressed by transfected cells, coelenterazine has to be added into culture medium to be taken up by the cells shortly before the experiment.

Aequorin assays were originally used only for Gq-coupled receptors due to their interaction with phospholipase C pathway. Nevertheless genetic expression methods enabled Ca^{2+} production upon activation of other GPCRs like Gi/o and Gs as well (Filmore, 2004). One of them is the AequoScreen® Technology by Perkin Elmer.

Other calcium assays usually share the same principle of the Ca^{2+} interaction with various fluorescent dyes (e.g. calcium assay kits compatible with the FLIPR® (**Fl**uorescent **I**maging **P**late **R**eader) Tetra system (Molecular Devices).

cAMP cell based assays are mostly based on competition between cAMP in cell lysate and variously labeled cAMP. In the cAMP AlphaScreen® assay (Perkin Elmer), stimulated cells are lysed with a specific buffer and mixed with labeled microbeads. There are two types of beads: e.g. a) so called donor beads are labeled with streptavidin and b) acceptor beads which are conjugated with anti-cAMP specific antibody. Upon lysis and addition of beads and biotinylated cAMP, a competition between endogenous and labeled cAMP occurs. When biotinylated cAMP is bound, a donor bead excitation with laser at 680 nm leads to the release of singlet of oxygen which travels up to 200 nm to the acceptor bead and causes an emission of light of the wavelength 520-620 nm (Performing alphascreen cAMP functional assay, PerkinElmer). In case the bound cAMP is unlabeled, there is no interaction between beads and no signal can be detected as the distance between the two types of microbeads is too big („Pic. 6”).



„Pic. 6": Simplified scheme of AlphaScreen® technology. The system consists of 3 basic components – Streptavidin-coated donor bead, anti-cAMP conjugated acceptor bead and biotinylated cAMP. After receptor stimulation a competition between exogenously added cAMP and intracellular cAMP occurs. The signal intensity changes in response to the cAMP levels in the cell (Self-made picture based on „Performing alphascreen cAMP functional assays", Perkin Elmer).

Another possibility for cAMP measurement is offered by the cAMP HTRF® assay (CisBio). HTRF stands for **H**omogeneous **T**ime-**R**esolved **F**luorescence technology, a method representing an enhanced FRET - **F**örster/**F**luorescent **R**esonance **E**nergy **T**ransfer based on the energy transfer between two chromophores. As one of the chromophores (called fluorescence donor) gets into an excited state, it may transfer part of its energy on the other chromophore (the fluorescence acceptor) if this one is located in close proximity of 1-7 nm (Grecco & Verveer, 2011). The donor is composed of anti-cAMP antibody and a cryptate together forming anti-cAMP cryptate conjugate. Cryptate stands for a complex formed by the inclusion of a cation into tridimensional cavity from synthetic cryptand (McNaught & Wilkinson, Compendium of chemical terminology 2nd edition 1997). In CisBio's HTRF assay Eu^{3+} trisbipyridine cryptate (TBP Eu^{3+}) is used. The advantage of this approach is the ability of the cryptate cage to act as light collecting device and relay the energy to the core of the cryptate - Eu^{3+}. The prolonged emission (300 µsec to 1 msec) after the excitation helps to improve the

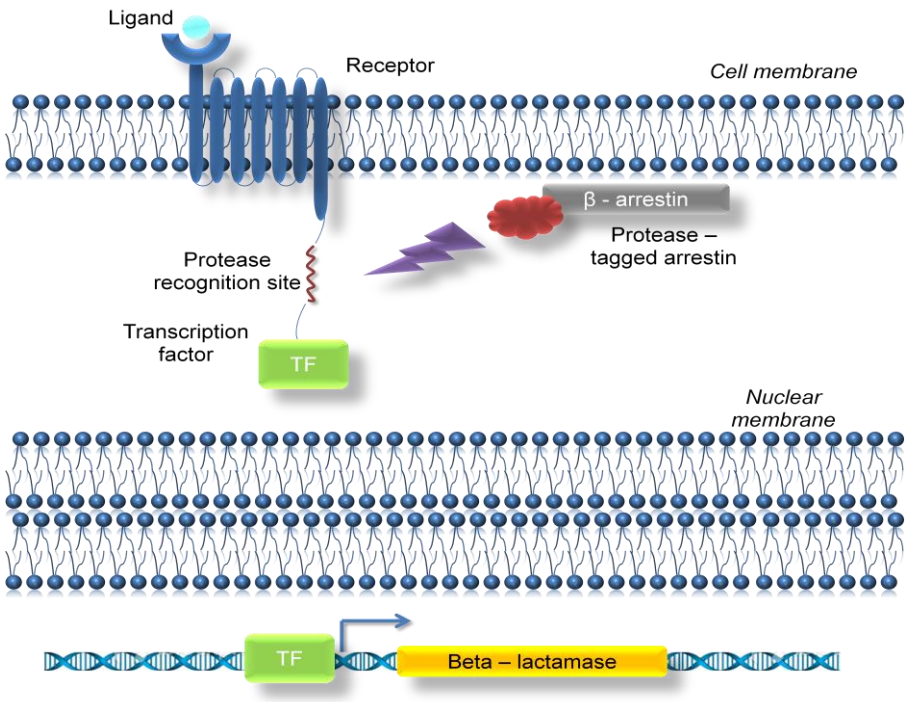
sensitivity of this assay, as several compounds from culture medium, serum, colored compounds or auto-fluorescent compounds may give a high background, HTRF emitted signal should be unmistakable. As the acceptor serves XL665, a crosslinked phycobiliprotein isolated from red Algae – allophycocyanin, or the second generation d2 acceptor, a 1000 Da organic compound (CisBio HTRF Methodological aspects, cAMP dynamic 2 manual).

The cAMP detection assays represent a tool predominantly for Gs or Gi/o coupled receptors. However, several difficulties occur during Gi/o receptor testing. Gs receptors enhance the activity of adenylyl cyclase, thus in response to receptor agonists cAMP levels increase. For Gi/o receptors the opposite is true – with the agonist binding cAMP level will decrease. But how can we actually measure something lower than low? As already mentioned, the effect of Gi/o coupled receptors is often connected to the activation of Gs coupled receptors. One of the possibilities how to solve this problem is to stimulate cells with forskolin - a labdane diterpene produced by a plant *Coleus forskohlii* able to activate adenylyl cyclase via interaction with the catalytic subunit (Seamon & Daly, 1981). Thus forskolin does not activate Gs-coupled receptor but directly stimulates the adenylyl cyclase activity, the final effect being higher cAMP level than those obtained with Gs-stimulation. Currently, it is more recommended to use calcitonin (George et al., 1997) – 32 amino acid polypeptide hormone. Calcitonin receptor is ubiquitously expressed and is coupled to Gs-protein. (Purdue et al., 2002). Activation of this receptor leads to an increase in cAMP levels and thus provides a more physiological response than direct adenylyl cyclase activation.

A more general approach for GPCRs investigation represents Tango® GPCR assay technology (Invitrogen). It is a useful tool when we do not have any knowledge about the coupling properties of our receptor, especially in the case of orphan receptors. It does not depend on any of the second messenger levels. Main principle consists in the process of receptor desensitization. Once a ligand is bound and receptor receives and transmits the signal, it is necessary to switch off the receptor to prevent persistent activation which should have destructive impact on the cell. A two-step process is involved: so-called GRKs (G-protein Coupled Receptor Kinases) phosphorylate serine and threonine residues in the carboxy terminal tail region and the third cytoplasmic loop of the 7TM to enable binding of β-arrestins – intracellular adapter proteins which sterically inhibit coupling to G-protein (Luttrell & Lefkowitz, 2002). β-arrestins bind very specifically only to the agonist activated and phosphorylated receptor. This process of silencing is called homologous desensitization. It means that even if the agonist is in

excess, the receptor does not respond to the signal because it is uncoupled from its signaling cascades (Reiter & Lefkowitz, 2006).

The Tango® cell-based assay requires special engineered cell lines containing the GeneBLAzer® beta-lactamasereporter system. These cells express an optimized beta-lactamase gene under the control of an exogenous transcription factor (TF) and need to be loaded with β-lactamase substrate (excitation/emission 409/520 nm, green signal) containing two different fluoroprobes - coumarin and fluorescein. The receptor C-terminus is fused to this exogenous TF using a specific sequence recognizable by non-native protease. This protease is tagged to the β-arrestins. When the ligand is bound and β-arrestins are recruited, the protease cleaves the sequence between the receptor and transcription factor which allows the TF to translocate into nucleus and switch on transcription of β-lactamase. Once the β-lactamase is expressed, it cleaves the substrate separating both fluorophores. When exited at 409nm, cleaved coumarine emits light with an emission maximum at 447nm compared to the fused coumarin-fluorescein complex that has its emission maxium at 520nm length. The agonist potential is expressed as the signal ratio (520nm/447nm) in response to the β-lactamase activity („Pic. 7“), (Tango® GPCR Assay Development Protocol, Invitrogen).



„Pic. 7”: Tango® GPCR GeneBLAzer® system. Specially engineered cell line expresses GPCR fused at the C-terminus to the exogenous transcription factor (TF) through the protease recognition site. The non-native protease is tagged to the β-arrestin. When receptor activation occurs, β-arrestins are recruited to stop signal propagation and the protease cleaves the

recognition site which allows TF translocation into the nucleus and beta-lactamase expression. A signal ration between substrate and coumarine fluorescence determines the agonist potential (Self-made picture based on „Tango GPCR Assay Development Protocol", Invitrogen).

Due to our actual knowledge about the Gi-coupling of GPR34 receptor referenced by Sugo et al., 2006 (Gi-coupled receptor, with necessary pre-stimulation) and due to the simplicity in comparison with e.g.Tango® GeneBLAzer® system, we decided to use CisBio's cAMP HTRF® assay for pharmacological characterication of GPR34. As a first step the generation of a cell line stably expressing GPR34 was required.

1.8 Approaches for integration into genome

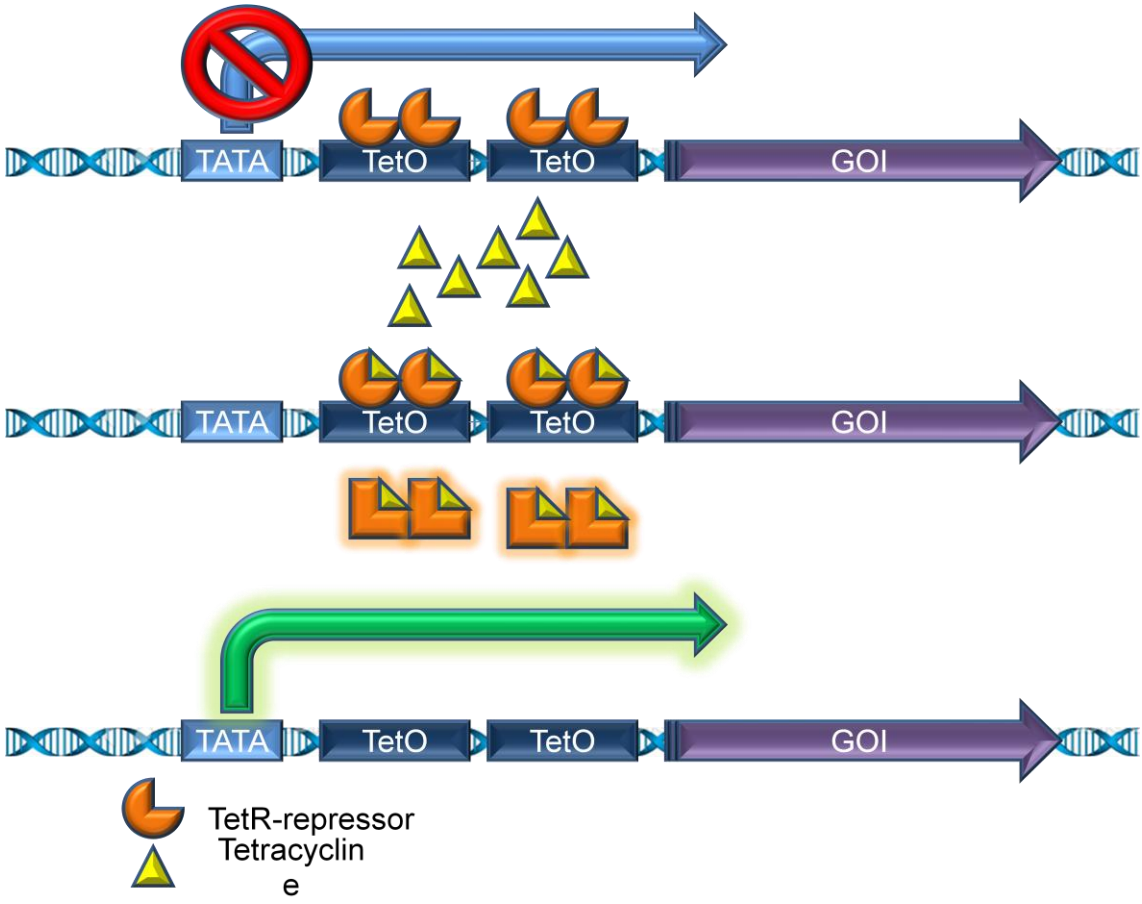
We decided to compare three different strategies of integration into the mammalian genome. The first one – classical gene delivery technique using Lipofectamine™ as the transfection reagent with random integration, then Jump-In™TI™Gateway® system (Invitrogen) based on the targeted integration and lastly Meganuclease Driven TI (Cellectis) based on homologous recombination between cell-native DNA sequences and engineered sequences.

Our gene of interest, human GPR34 is expressed with N-terminal FLAG®-tag (Sigma-Aldrich). FLAG®-tag is an octapeptide chain (N - Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys - C) which is being use as an expressed epitope for versatile protein labeling (Einhauer & Jungbauer, 2001). Detection of the tagged protein is possible with immuno-based methods using anti-FLAG antibodies.

Moreover we decided to combine these techniques with T-REx™ system (**T**etracycline-**R**egulated **Ex**pression System, Invitrogen) which enables expression of the GOI under the control of inducible promoter („Pic. 8"). This is very useful when we investigate an unknown receptor whose expression could be lethal for cells. The promoter is a standard CMV promoter (**C**yto**m**egalovirus **P**romoter) with two tetracycline operator sequences ($TetO_2$) inserted in tandem. The system works with T-REx™ cell lines which express the tetracycline-inducible repressor protein (TetR). These cell lines are commercially available or may be created by user from any cell line of choice by transfection with TetR containing vector e.g. pcDNA6/TR© (Invitrogen).

The main principle of this inducible expression system consists in blocked transcription in the absence of tetracycline because the TetR protein homodimers bind to the operator sequences in promoter region preventing the GOI from being transcripted. Upon tetracycline addition, it binds to the repressor which changes its conformation

and the whole complex dissociates from the promoter allowing normal transcription (in a reversible manner) (Gossen & Bujard, 1992).



„Pic.8": Tetracycline-regulated expression system. Gene of interest (GOI) is under the control of tetracycline inducible promoter. In the absence of tetracycline, repressor binds to the regulatory sequence in the promoter region and no transcription occurs. After tetracycline addition, it binds to the repressor homodimers which change conformation and allow the GOI to be expressed (Self-made picture based on „T-Rex system manual", Invitrogen).

Random House´s Webster´s Unabridged Dictionary from 1997 defines transfection as „the insertion into a cell of a bacterial plasmid that contains a foreign virus or genetic material." A newer definition is more specific: „Transfection is a process of nucleic acid introducing into eukaryotic cells using nonviral delivery methods" (Protocols & Applications Guide, Promega, rev. 3/11). Transfection techniques can be divided into chemical, physical and usage of cationic lipids, depending on the way which is being used to overcome the plasma membrane barrier. General rule is that the transfection is only temporary and that after some time the introduced genetic material is degraded by intracellular processes. When our intent is to prepare cells that only transiently

expressing our GOI, the result is pretty satisfactory and we can observe high expression during 2-10 days post-transfection (Baldi et al., 2007). Disadvantage is poor reproducibility and only transient expression which cannot be transfer onto the next generation.

Randomly, although this is happening rather seldom, the introduced DNA gets to the cell nucleus and is permantely inserted into the genome. Moreover into a part of genome which is being translated and which does not hit any important sequences essential for normal cell function. We can put our GOI under the control of more „aggressive" promoter but we cannot control the whole process at all. The actual need of more controlled processes together with transfection reproducibility and HTS compatibility led to the development of more sophisticated methods based on directed integration into specific genomic locations.

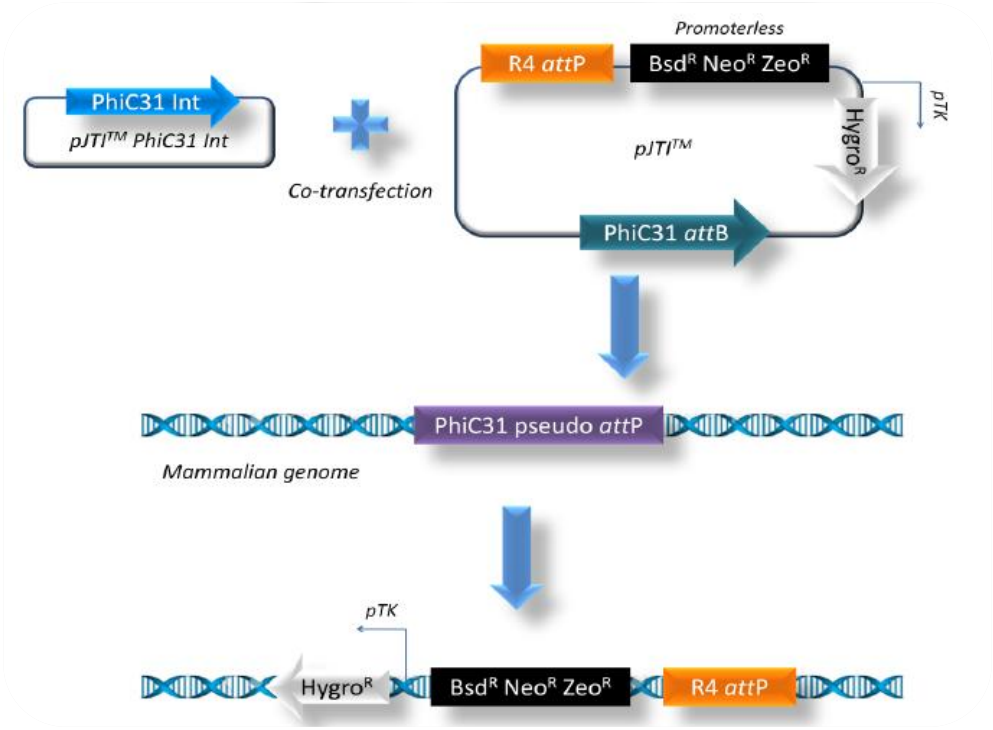
1.8.1 Jump-In™ TI™ Gateway® System (Invitrogen, USA)

Jump-In™ TI™ Gateway® is a system based on site-specific recombination catalyzed by two specific recombinases. Site-specific recombination was discovered in bacteria and yeast as a naturally occurring process e.g. the bacteriophage insertion into the host genome. As it follows from the name, the insertion is not random but it directed by the presence of specific sequences or recognition sites. Mechanism of DNA recombination involves DNA breakage and reunion with no need of DNA synthesis or presence of high-energy factors like ATP (Grindley et al., 2006).

The Jump-In™ system, as a part of Jump-In™ TI™ Gateway®, is based on the use of two specific enzymes: PhiC31 integrase and R4 integrase, belonging to a family of serine-recombinases. The group is named after the amino acid residue within the catalytic domain responsible for a protein-DNA linkage in the reaction intermediate (Wang et al., 2011). Enzymes from this group specifically recognize DNA sequences, traditionally referred as *att*B and *att*P sites, which are formed by recombination into hybrid sites called *att*R and *att*L. Compared to tyrosine recombinases which act through sequential breaking and rejoining of single strands (through the Holliday-Junction intermediate), serine-recombinases cut all strands in one single step. This reaction is unidirectional and virtually irreversible due to the lack of a corresponding excisionase.

The main principle lies in the insertion of a specific sequence into the DNA which serves as the integration site for retargeting – introducing the main DNA sequence

containing our GOI. In a first experimental step we have to create so-called platform cells lines or master cell lines which are further used for retargeting. This process consists in the co-tranfection of two vectors – pJTI^{TM} platform vector and pJTI^{TM} PhiC31 Int vector which expresses the PhiC31 integrase („Pic. 9"). The platform vector contains PhiC31 *att*B recognition site, R4 *att*P retargeting site, hygromycin-resistance gene and promoterless gene for the second selection agent. Recombination event occurs between *att*B site in the pJTI^{TM} platform vector and PhiC31 pseudo *att*P site naturally present in the genome. The number of pseudo *att*P sites may reach up to 24 sites per genome and they are all transcriptionally active. Positive clones are selected due to the resistance to hygromycin and should be tested for presence and number of retargeting sequences (Jump-In^{TM} TI^{TM} Gateway® system manual, Invitrogen).

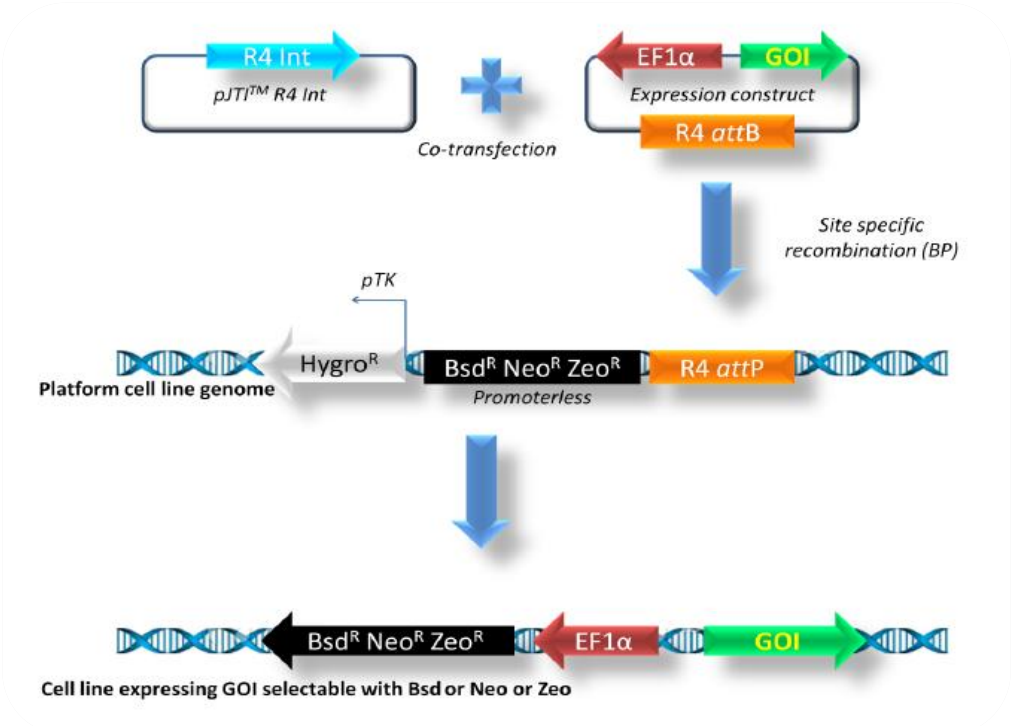


„Pic. 9": Jump-In^{TM} TI^{TM} Gateway® system – platform cell line generation. Co-transfection of two vectors – PhiC31 integrase expressing and so-called platform vector is performed. Site specific recombination between PhiC31 *att*B site in the platform vector and PhiC31 pseudo *att*P site catalyzed by PhiC31 integrase leads to the whole vector integration into the genome (Self-made picture based on „Jump-In^{TM} TI^{TM} Gateway® system manual", Invitrogen).

The second step is the retargeting of previously selected and characterized platform cell lines. Retargeting happens via co-transfection of two vectors pJTI^{TM} R4 Int expressing R4 integrase and the so-called expression construct. An expression construct is generated from pJTI R4 DEST vector using Gateway® (further developed to MultiSite Gateway®) technology which allows simultaneously clone up to 4 different

DNA fragments in one destination vector and their delivery to target genomic DNA. The whole system works like a jigsaw with subsequent recombination steps. First all DNA fragments have to undergo a PCR reaction with site-specific primers in order to have the products flanked with different *att*B recombination sites. Second we have to clone these products into corresponding pDONR vectors to obtain Entry Clones. This reaction is performed *in vitro* and is catalyzed by a so-called BP-reaction mixture (composition is undisclosed by Invitrogen, USA). The third step is the site-specific recombination which arranges all the fragments into the selected destination vector to obtain the Expression Clone which is further used for retargeting. This reaction is catalyzed by so-called LR-recombination mixture.

The final expression construct is used for platform cell line retargeting by co-transfection with R4 integrase expressing vector, when site-specific recombination between *att*B sites (present in the expression construct) and *att*P sites (previously introduced into the genome) occurs („Pic. 10") (Jump-In™ TI™ Gateway® system manual, Invitrogen), (Multisite Gateway® Pro manual, Invitrogen).



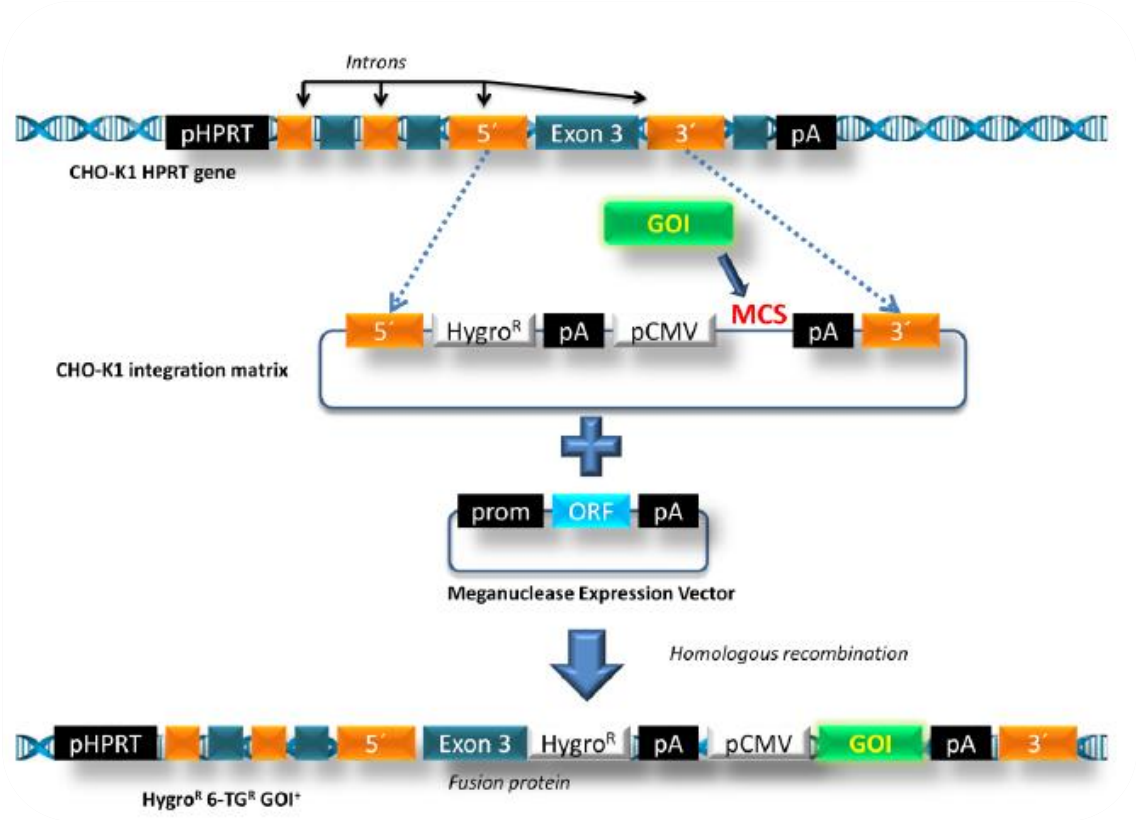
„Pic. 10": Platform cell line retargeting. Co-transfection of R4 integrase expressing vector and expression construct leads to site-specific integration into the previously introduced *att*P sites. Succesful integration switches on the gene for resistance to blasticidin or neomycin or zeocin which enables to isolate positive population (Self-made picture based on Jump-In™ TI™ Gateway® system manual; Invitrogen, USA).

1.8.2 cGPS® (Cellular Genome Positioning System) Meganuclease – driven targeted integration (Cellectis Bioresearch, France)

Meganucleases are sequence-specific DNA endonucleases naturally occurring in yeast or bacteria with long recognition sites (12-30 bp) which induce double-strand breaks and subsequent homologous recombination. The basic principle of this DNA integration method consists of co-transfection of Meganuclease-expressing vector and a delivery vector called Integration matrix containing the GOI, promoterless gene for hygromycin and two regions homologous to those present at the targeted locus. The targeted loci used in the meganuclease approach are the HPRT (Hypoxanthine-guanine phosphoribosyl transferase) gene in CHO cells, the human RAG1 gene (Recombination Activating Gene 1) for most of the human derived cell lines or LP1A-site (Landing Pad) for engineered cell lines containing this integration site (Cellectis Bioresearch, France).

Targeting of the cell lines consists of one transfection step where both vectors are being introduced into the cell. Once specially engineered meganuclease is being expressed; it recognizes the HPRT sites and induces double strand break. The cell senses the DNA damage and initiates homologous recombination using the integration matrix to fix the DNA breakage (due to the presence of HPRT regions).

Successful integration switches on the hygromycin resistance gene (expressed under HPRT promoter as a fusion protein with the first exons of HPRT) („Pic. 11”). Inactivation of HPRT also leads to resistance to 6-thioguanine (6-TG). Stable population can be than selected for the double resistance – hygromycin/6-TG. A prerequisite condition is that the cell line is monoallelic in HPRT and also the meganuclease had to be especially engineered by Cellectis Bioresearch for various cell lines.



„Pic. 11": Meganuclease – driven targeted integration. co-transfection step of the meganuclease expressing vector and GOI containing vector (so-called integration matrix) leads to the DNA cleavage and activation of the fixing mechanism based on the homologous recombination with the use of integration matrix (Self-made picture based on „cGPS Custom CHO-K1 Full Kit", Cellectis, France)

2 Experimental part

2.1 Material

Cells and their supplies

CHO#35 T-Rex cells (parental cell line derived from CHO-K1 cells, generated in-house at Amgen, Regensburg); One Shot® Mach1^{TM} – T1^{R} Chemically Competent *E. coli* (Invitrogen, USA); One Shot® ccdB Survival^{TM} 2 T1^{R} Chemically competent cells (Invitrogen, USA); ElectroMAX™ DH5α-E™ Competent Cells (Invitrogen, USA); S.O.C. medium (Invitrogen, USA); LB-agar selective plates (100 µg/ml ampicilin or 50 µg/ml kanamycin); LB-medium; Lipofectamine^{TM} 2000 reagent (Invitrogen, USA); PolyFect^{TM} Transfection reagent (Qiagen, Netherlands); Opti-MEM® (Reduced Serum Medium, Invitrogen, USA); 1xPBS (Gibco® Dulbecco´s Phosphate buffered saline, pH=7,0-7,2 Ca^{2+}(-), Mg^{2+}(-), Invitrogen, USA)

DNA and RNA equipment

TRIzol® reagent (Invitrogen, USA); RNase *AWAY*^{TM} reagent (Invitrogen, USA); DEPC treated H_2O; Chloroform (Merck, Germany); Isopropanol (Merck, Germany); 75% Ethanol (Merck, Germany); 10x PCR buffer 15 mM $MgCl_2$ (Qiagen, Netherlands); 25 mM $MgCl_2$ (Qiagen, Netherlands); 10 mM dNTP mix (Qiagen, Netherlands); Taq DNA Polymerase 5U/µl (Qiagen, Netherlands); Trypsin/EDTA (Gibco, USA); 100% EtOH (Merck, Germany); GeneRuler^{TM} 1 kb DNA Ladder (Fermentas, USA); O´RangeRuler^{TM} 100 bp DNA Ladder (Fermentas, USA); 6x Orange DNA loading dye (Fermentas, USA); Agarose electrophoresis grade (Sigma-Aldrich, USA)

Buffers and solutions:

1x TE buffer pH=8 (10 mM Tris-Cl, 1 mM EDTA); 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH=8); Maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH=7,5); 1x Washing buffer (100 mM Maleic acid, 150 mM NaCl, 0,03 % (v/v) Tween 20); 1x Detection buffer (100 mM Tris-Cl, 100 mM NaCl, pH=9,5 20x SSC (3 M NaCl, 0,3 M Trisodiumcitrate, pH=7); 1x Denaturation solution (1,5 M NaCl, 0,5 M NaOH); 1x Neutralization solution (1,5 M NaCl, 0,5 M Tris-Cl, pH=7); 3 M sodium acetate (CH_3COONa, NaOAc) pH=5,2; 0,2 M EDTA, pH=8; 10% SDS solution (w/v); 0,2 mM EDTA in 1xPBS; dH_2O; 0,5% glutaraldehyde (Sigma-Aldrich, USA) in 1xPBS; 1x PBS/0,01% Pluronic® F-68 (Invitrogen, USA)

Other chemicals:

6-thioguanine (Sigma-Aldrich, USA); X-Gal staining solution: 20 mM potassium ferrocyanide ($K_4Fe(CN)_6$), 20 mM potassium ferricyanide ($K_3Fe(CN)_6$), 2 mM $MgCl_2$, 1xPBS, pH=7,8, just before use was added 100 µl of X-gal solution (50 mg/ml, Promega, USA) per every 5 ml of the solution being used; Forskolin 10 mM stock in DMSO (Carl Roth, Germany); Calcitonin salmon 100 mM in 1% acetic acid (Sigma Aldrich, USA); Proteinase K solution (Invitrogen, USA)**;** BP Clonase™ II enzyme mix (Invitrogen, USA); LR Clonase™ II enzyme mix (Invitrogen, USA)

Restriction endonucleases and their accessories

New England Biolabs (USA): *Sma*I*; Eco*RV; *Cla*I; *SnaB*I; *BamH*I; *Nde*I; *Sca*I HF; *Hind*III; *Xba*I; BSA 10 mg/ml; 10x NEBuffer 2; 10x NEBuffer 4

Fermentas (USA): *Nru*I; 10x Buffer O+; Shrimp Alkaline Phosphatase 1000 U/ml (SAP)

Antibodies:

Primary antibodies (AB1): monoclonal anti-flag M2 produced in mouse (Sigma-Aldrich, USA), mouse anti-IgG_1 (Calbiochem – Merck Group, Germany); secondary antibody (AB2): FITC labeled goat anti-mouse (DakoCytomation, Denmark)

Antibiotics:

Hygromycin-B (Invitrogen, USA); Zeocin (Invitrogen, USA); Blasticidine (Invitrogen, USA); Tetracycline (Invitrogen, USA); Ampicilin solution 100 mg/ml (Sigma-Aldrich, USA); Kanamycin solution 50 mg/ml (Sigma-Aldrich, USA)

Tested compounds:

All tested compounds were purchased from Sigma-Aldrich (USA).

L-α-Lysophosphatidylinositol sodium salt from soybean (Cat. Nr. 62966); 3-sn-Phosphatidylethanolamine from bovine brain (Cat. Nr. P7693); Arachidonic acid (Cat. Nr. A3555); 1,2-Diacyl-sn-glycero-3-phospho-L-serine (Cat. Nr. P6641); 1-palmitoyl-sn-glycero-3-phospho-l-serine (Cat. Nr. 51581); L-α-Lysophosphatidylcholine from bovine brain (Cat. Nr. L1381); L-α-Lysophosphatidylcholine from egg yolk (Cat. Nr. L4129); Sphingosylphosphorylcholine (Cat. Nr. S4257); Sphingosine 1-phosphate (Cat. Nr. S9666)

Kits:

QIAfilter Plasmid Maxi Kit (Qiagen, Netherlands); QIAprep Spin Miniprep kit (Qiagen, Netherlands); MinElute Gel Extraction Kit (Qiagen, Netherlands); SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, USA); QIAamp DNA Mini Kit (Qiagen, Netherlands); DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Germany); Rapid DNA Ligation Kit (Roche, Germany); cAMP dynamic kit (Cisbio, France)

Vectors:

pJTI™ PhiC31 Int vector DNA (Invitrogen, USA); pJTI™/Zeo platform vector DNA (Invitrogen, USA); pcDNA5/TO vector c=1 µg/µl (Invitrogen, USA); hGPR34 in pFLAG-CMV-3 c=1 µg/µl (initial construct); pDONR™ 221 P1-P5r vector DNA 50 ng/µl; pDONR™ 221 P5-P2 vector DNA 50 ng/µl; pJTI™ R4 DEST vector 50 ng/µl; pcGPS-C.CHO.MCS (Integration matrix) (Cellectis, France); pcDNA5/TO/LacZ β-galactosidase expressing control plasmid (Invitrogen, USA); pcGPS-C.CHO.MEGA (Meganuclease expressing plasmid) (Cellectis, France); pcGPS-C.CHO.LacZ (LacZ containing control integration matrix) (Cellectis, France); pJTI™ R4 Integrase expressing vector (Invitrogen, USA); pENTR™ L1-pLac-*lac*Zα-R5 (Invitrogen, USA), pENTR™ L5-pLac-Spec-L2 (Invitrogen, USA)

Primers:

β-actin (+) sense primer (designed by Amgen, synthesis by Invitrogen); β-actin antisense (-) primer (designed by Amgen, synthesis by Invitrogen); *att*P (+) sense primer (Self-designed, produced by GeneArt); Zeo (-) antisense primer (Self-designed, produced by GeneArt); *att*B1-pCMV/TO sense primer (Self-designed, produced by GeneArt); *att*B5r_pCMV/TO antisense primer (Self-designed, produced by GeneArt); *att*B5_pFLAG-hGPR34-BGH pA sense primer (Self-designed, produced by GeneArt); *att*B2_pFLAG-hGPR34 BGH pA antisense primer (Self-designed, produced by GeneArt)

Culture medium:

<u>Culture medium CHO#35 T-Rex cells</u>

DMEM/F-12 (GIBCO® Dulbecco´s Modified Eagle Medium: Nutrient mixture F-12, Invitrogen, USA) with 10 % FBS (Dialyzed Fetal Bovine Serum, Pan Biotech, Germany), 5 % P/S (Pennicilin/Streptomycin, Gibco, USA), 5 % L-Gln (L-Glutamine, Gibco, USA) and 6 µg/ml blasticidine (Invitrogen, USA)

Transfection medium

DMEM/F-12, 10 % FBS, 5 % P/S, 5 % L-Gln

Selection medium

- Platform CHO#35 T-Rex Zeo platform cells and convetionally transfected cells with pcDNA5/TO or pcDNA5/TO/LacZ
  DMEM/F-12, 10 % FBS, 5 % P/S, 5 % L-Gln, 6 µg/ml blasticidine, 600 µg/ml hygromycin
- Jump-In TI Gateway retargeted cells
  DMEM/F-12, 10 % FBS, 5 % P/S, 5 % L-Gln, 6 µg/ml blasticidine, 150 µg/ml zeocin)
- Meganuclease retargeted cells
  DMEM/F-12, 10 % FBS, 5 % P/S, 5 % L-Gln, 6 µg/ml blasticidine, 600 µg/ml hygromycin, 6 µg/ml 6-thioguanine

Special additional equipment:

BioRad GenePulser cuvette 0,1 cm (BioRad, USA); Whatman 3 MM paper 20 x 20 cm (Whatman, UK); Positively charged nylon membranes, 10 x 15 cm (Roche, Germany); 5 ml FACS polystyrene round bottom tubes (Becton, Dickinson and Company, USA); Clear 384-well round bottom polypropylene plates (Corning, USA); White 384-well round bottom polystyrene microplates (Greiner Bio-one, Germany)

Special instrumental equipment:

BioRad MicroPulser (BioRad, USA); Hybaid PCR Sprint Thermal Cycler (Thermo Scientific, USA); Fusion FX7 imager (Vilber Lourmat, France); CyBi®-Well 96- and 384- Channel Simultaneous Pipettor (CyBio, Germany); Multidrop Combi Reagent Dispenser (Thermo Scientific, USA); EnVision Microplate reader (Perkin Elmer, USA); Axiovert 200M Microscope (Zeiss, Germany); FACS Aria (BD Bioscience, USA)

Software equipment:

Vector NTI Advance™ 11 (Invitrogen, USA)

GraphPad Prism 5 (GraphPad Software, USA)

2.2 Methods

2.2.1 Cell culture

The parental cell line CHO#35 T-Rex stably expressing aequorin protein and tetracycline repressor protein was cultured in DMEM/F-12 Medium with 10 % FBS, 5 % P/S, 5 % L-Gln and 6 µg/ml blasticidine. Cells were kept in incubator in humidified atmosphere at 37°C, 5 % CO_2. For transfections we used DMEM/F-12 (GIBCO® Dulbecco´s Modified Eagle Medium: Nutrient mixture F-12, Invitrogen) with 10% FBS, 5% P/S, 5% L-Gln as cultivation medium.

2.2.2 Transformation of competent cells

One Shot® Mach1™ – T1^R Chemically Competent *E. coli* (Invitrogen) was used for most of the plasmid preps if not otherwise mentioned. Competent cells were transformed as recommended. Briefly, bacterias were thawed on ice and 1 µg of purified plasmid was added into 1 vial. Vials were gently mixed and incubated on ice for 30 min. Cells were than heat-shocked for 30 sec in 42°C bath and placed on ice for another 2 minutes. 250 µl of S.O.C. medium (Invitrogen) was added into vial and incubated horizontally for 1 hour at 37°C in shaking incubator (200 rpm). Two aliquots of bacterias (25 µl and 50 µl) were than plated on pre-warmed LB-agar selective plates with either ampicilin or kanamycin and incubated overnight at 37°C. Next day, a single colony was picked and transferred into LB medium containing either 100 µg/ml ampicilin or 50 µg/ml kanamycin and cultured overnight at 37°C in shaking incubator (200 rpm).

One Shot® ccdB Survival™ 2 T1^R Chemically competent cells (Invitrogen) were handled with the same manner as previously described. These competent cells are resistant to the toxicity of *ccd*B gene and are used to propagate and maintain vectors containing this gene e.g. pDONR™ 221 vectors and pJTI™ R4 DEST vector from the MultiSite Gateway® system. Moreover the *ccd*B gene is being used as the one of the selection markers for succesful recombinational steps as it is removed during site-specific recombination. If the recombination is unsuccesful and products from recombination event are propagated using unresistant cells, no colonies appear.

ElectroMAX™ DH5α-E™ Competent Cells (Invitrogen) are electrocompetent cells which were used for handling with Meganuclease integration matrix due to their ability to propagate plasmids up to 30 kb (Invitrogen). 1 µg plasmid DNA was added to 50 µl of competent cells and the whole mix was transferred into BioRad GenePulser cuvette 0,1 cm. The cuvette was placed into BioRad MicroPulser and a single pulse (1,8 kV) was used for electroporation. Bacterias were washed out of the cuvette with 250 µl of

LB medium and placed onto a shaking incubator for 1,5 hours at 200 rpm and 37°C. Two aliquots (25 µl and 50 µl) were than plated on pre-warmed selective plates and incubated overnight at 37°C. Next day, a single colony was picked and transferred into LB medium (containing appropriate antibiotic) and cultured overnight at 37°C with shaking (200 rpm).

2.2.3 Plasmid purification

QIAfilter Plasmid Maxi kit (Qiagen) (all buffers and other components are part of this kit). Cells from large overnight culture (200 ml) were harvested and centrifuged for 20 min, at 4000 rpm and 4°C. Supernatant was poured off and pellet was resuspended in 10 ml of Buffer P1. Cells then underwent an alkaline lysis due to addition of buffer P2 (10 ml). After buffer addition tubes were mixed by inversion and incubated for 5 min at RT. Samples were neutralized by addition of 10 ml of pre-chilled buffer P3 and mixed by inverting. Lysates were poured into the QIAGEN-cartridge and incubated for 10 min at RT. After incubation were lysates filtered into previously equilibrated QIAGEN-tips (washed with 10 ml QBT buffer). Filtered lysates were allowed to come through the column by gravity flow. Columns with bound samples were 2 x washed with 30 ml of buffer QC and eluted with 15 ml of elution buffer QF. Collected samples were precipitated by addition of 10,5 ml isopropanol (RT) and centrifuged for 30 min at 4100 rpm and 4°C,. Supernatant was subsequently decanted, and the DNA pellet was resuspended in 70% EtOH (RT) and samples were once more centrifuged for 15 min at 4100 rpm and RT. Supernatant was removed by pipetting and pellets were air-dried. DNA was redissolved in 400 µl of dH_2O and concentration was measured spectrophotometrically (λ=260 nm, conversion factor 1AU=50 µg/ml)

QIAprep Spin Miniprep kit (Qiagen) (this protocol was used for small overnight cultures 5-10 ml). Cells were centrifuged for 20 min at 4000 rpm and 4°C and pellets were resuspended in 250 µl buffer P1. Samples were lyzed by addition of 250 µl buffer P2 (incubation 5 min, RT) and neutralized with 350 µ buffer N3. Lysates were centrifuged for 10 min at 13000 rpm and 4°C. Supernatants were isolated and after resuspension applied onto QIAprep spin columns. Samples were centrifuged for 1 min at 13000 rpm and RT. Flow-through was discarded and the spin columns were washed by addition 500 µl buffer PB and centrifuging for 1 min at 13000 rpm and RT. Flow-through was discarded and columns washed by addition 750 µl buffer PE and centrifuging for 1 min at 13000 rpm and RT. Flow-through was discarded and columns one more spinned to remove residual wash buffer. Columns were then placeed in a clean microcentrifuge tube and filled with 25 µl dH_2O. Samples were incubated for 1

min at RT and subsequently centrifuged for 1 min at 13000 rpm and RT to elute purified DNA samples from columns.

2.2.4 Creation of platform cell lines

One day prior to the experiment CHO#35 T-Rex cells were (3rd passage) seeded to become ~ 80% confluent after a 24h incubation (1.10^6 cells for Ø 14,5 cm culture Petri dish). One hour prior to the transfection culture medium was removed, and cells were washed once with 1xPBS and transfection medium was added (omits blasticidine). 33 µg of pJTI™ PhiC31 Int vector and 33 µg of pJTI™/Zeo platform vector were diluted in 4,125 ml of Opti-MEM®. 165 µl of Lipofectamine™ 2000 reagent was gently mixed, added into 4,125 ml Opti-MEM® and incubated for 5 min at RT. After incubation, the diluted DNA was mixed with lipofectamine and incubated for another 20 min at RT. The whole mixture was added dropwise to the plated cells and incubated for 4-6 hours at 37°C in atmosphere of 5 % CO_2. After incubation, medium was removed and cells were once washed with 1xPBS and fresh transfection medium added to the cells. On the next day cells were switched to the selection medium.

2.2.5 RNA isolation using Trizol reagent

One day prior to the experiment CHO#35 T-Rex Zeo platform cells were seeded onto the large Petri dishes (Ø 14,5 cm) at a density allowing them to become almost confluent the following day (seeding 1.10^6 cells per dish). The medium was removed from cells and 10 ml of Trizol reagent was added to each dish. Cells were gently scrapped of the surface of the dish and transfered into 50 ml falcon tube. Samples were incubated for 5 min at RT. 2 ml of chloroform was then added and samples were hand-shaken for 15 sec and incubated for 3 min, RT. Tubes were centrifuged for 15 min at 4100 rpm and 4°C. The aequos phase containing RNA was carefully collected and transferred into a fresh tube. RNA was precipitated by addition of 5 ml isopropanol. Samples were incubated for 10 minutes at RT and subsequently centrifuged for 10 min at 4100 rpm and 4°C. Supernatant was removed via a pipette and pellets were washed with 5 ml of 75% EtOH. Samples were mixed by vortexing and centrifuged for 5 min, at 4100 rpm and 4°C. Supernatant was removed and pellets were left to air-dry. Pellets were resuspended in 100 µl DEPC-treated H_2O and incubated for 10 min at 60°C and samples were stored until usage in a freezer at -80°C.

2.2.6 RT – PCR

First strand cDNA synthesis

Samples were prepared as described in the „Tab.1“ in 0,2 ml PCR tubes. Tubes were incubated for 5 min at 65°C and then placed on ice. All experiments were preformed in the presence of a positive control (with control RNA) and negative control with sample RNA but without subsequent addition of SuperScript™ II Reverse Transcriptase.

„Tab. 1“: Sample mixtures composition. Each component states per one tube.

Component	Sample	(+) Control	(-) Control
Total RNA	0,7 µg	-	0,7 µg
Control RNA (50 ng/µl)	-	1 µl	-
10 mM dNTP mix	1 µl	1 µl	1 µl
Oligo(dT)$_{12-18}$ (0,5µg/µl)	1 µl	1 µl	1 µl
DEPC-treated H_2O	up to 10 µl	up to 10 µl	up to 10 µl

Meanwhile was prepared reaction mixture as decribed in „Tab. 2“.

„Tab. 2“: Reaction mixture composition.

Component	Per reaction
10x RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0,1 M DTT	2 µl
RNaseOUT (RNase inhibitor)	1 µl

9 µl of reaction mixture was added per sample mixture tube, briefly mixed, collected by centrifugation and incubated for 2 min at 42°C. 1 µl (50 units) of reverse transcriptase was added to each tube except to the no RT control, mixed and incubated for 50 min at 42°C. Reverse transcriptase reaction was terminated for 15 min at 70°C and samples were subsequently chilled on ice. 1 µl of Rnase H was added to each tube and incubated for 20 min at 37°C.

cDNA amplification

Reaction mixture for each sample was prepared as described below („Tab. 3“).

„Tab. 3“: PCR mixture for cDNA amplification

Component	Per reaction
cDNA from the first reaction	2 µl
10x PCR buffer 15 mM $MgCl_2$	5 µl
25 mM $MgCl_2$	1,5 µl
10 mM dNTP mix	1 µl
10 µM attP (+) primer	1 µl
10 µM Zeo (-) primer	1 µl
10 µM β-actin (+) primer	0,2 µl
10 µM β-actin (-) primer	0,2 µl
Taq DNA Polymerase (5U/µl)	0,4 µl
PCR H_2O	36,7 µl
Total reaction volume	50 µl

Into the tube with positive control 1 µl of control primer A (10 µM) and 1 µl of control primer B (10 µM) were added instead of *att*P (+) and Zeo (-) primers.

Samples were gently mixed and placed in a thermal cycler. PCR was set up as described in „Tab. 4“.

„Tab. 4“: PCR set up for cDNA amplification.

PCR setup	Temperature	Time	
First denaturation	94°C	4 min	
Denaturation	94°C	30 sec	35x
Annealing	60°C	30 sec	
Elongation	72°C	60 sec	
End elongation	72°C	10 min	
Cooling	4°C		

After the PCR 10 µl of each sample was analysed using agarose gel electrophoresis.

2.2.7 DNA extraction from agarose gel

DNA fragments were extracted from the gel with scalpel and the weight of the gel fragments was determined. After this we added three volumes of buffer QG to the gel (e.g. 300ul of buffer for each 100ug of gel). Samples were incubated at 50°C for 10 min. One gel volume of isopropanol was added to the mixture and tubes were mixed by inverting the tubes multiple times. Samples were applied to MinElute columns and centrifuged for 1 min at 13000 rpm and RT. Flow-through was discarded, and 500 µl of QG buffer was added and tubes were centrifuged as in the previous step. Flow-through was discarded and 750 µl of buffer PE was added to the column and tubes were centrifuged as previously. Again the flow-through was discarded and tubes were one more centrifuged with the same parameters. The MinElute column was placed into a

clean 1,5 microcentrifuge tube and 20 µl of dH_2O was added. Samples were incubated for 1 min and subsequently centrifuged for 1 min at 13000 rpm and RT to elute DNA from the column.

2.2.8 Genomic DNA isolation

One day prior to the experiment CHO#35 T-Rex Zeo platform cells were seeded in large Petri dishes (Ø 14,5 cm) at 1.10^6 cells per dish. Medium was removed and cells were once washed with 1xPBS and harvested after trypsinization. Cells were then mixed with equal volume of culture medium and centrifuged for 5 min at 800 rpm and RT. Supernatant was removed, and the cell pellet was resuspended in 200 µl PBS. 20 µl of proteinase K was added to each sample. 200 µl of buffer AL was added per sample and tubes were vortexed for 15 sec. Samples were then incubated for 10 min at 56°C. After incubation tubes were briefly centrifuged and 200 µl of EtOH was added. Samples were mixed by vortexing (for 15 sec) and briefly centrifuged. Samples were then applied to QIAamp Spin Column and centrifuged for 1 min at 8000 rpm and RT. Columns were placed in a clean collection tube, washed with 500 µl buffer AW1 and centrifuged as in the previous step. Columns were placed to a new collection tube, filled with 500 µl buffer AW2 and centrifuged for 3 min at 13000 rpm and RT. Spin columns were then replaced in a clean microcentrifuged tube and 100 µl of water was added. Samples were incubated for 5 min at RT and subsequently centrifuged for 1 min at 8000 rpm and RT.

2.2.9 Southern Blot

Southern blot analysis was used to detect the presence and number of integration sites in the platform cell line genome.

Probe labeling

A 520 bp fragment of the pJTI™/Zeo platform vector was selected as a probe for the Southern blot analysis. This sequence is a part of the hygromycin resistance gene region obtained by restriction with *Nde*I (713) and *Sca*I (1233) restriction endonucleases.

Total 100 µg of pJTI/Zeo plasmid DNA was cut overnight at 37°C using *Nde*I and *Sca*I HF in NEBuffer 4 and analysed using gel electrophoresis (1% agarose in 1xTAE, 70 V). Appropriate bands with a length of 520 bp were isolated from the gel as previously described. 3000 ng of the fragment DNA (11,43 µl, filled with dH_2O up to 16 µl) was boiled for 10 min and quickly chilled on ice. 4 µl of DIG-High Prime mixture was added to the denaturated DNA and briefly centrifuged. Sample was incubated O/N at 37°C.

The labeling reaction was stopped by addition of 2 µl 0,2 M EDTA and heating to 65°C for 10 min.

Determination of labeling efficiency

DIG-labeled control DNA was diluted to 1 ng/µl in a DNA dilution buffer (from DIG system) and a dilution series was prepared as described below („Tab. 5“). The labeled probe was diluted in the same manner.

„Tab. 5”: Dilution series of the labeled and DIG-labeled control DNA.

Tube	DNA (µl)	From tube #	Dilution buffer (µl)	Dilution	Final c
1		diluted original			1 ng/µl
2	2	1	198	1:100	10 pg/µl
3	15	2	35	1:3,3	3 pg/µl
4	5	2	45	1:10	1 pg/µl
5	5	3	45	1:10	0,3 pg/µl
6	5	4	45	1:10	0,1 pg/µl
7	5	5	45	1:10	0,03 pg/µl
8	5	6	45	1:10	0,01 pg/µl
9	0	-	50	-	0

1 µl drop of each tube 2-9 of control DNA and self-labeled DNA was dropped on the dry piece of nylon membrane. Samples were fixed to the membrane by baking for 30 min at 120°C. Membrane was placed into 20 ml maleic acid buffer and incubated while shaking for 2 min at RT. Membranes were then: incubated for 30 min in 20 ml blocking solution, for 30 min in 10 ml antibody solution (1:10000), washed 2 x 15 min with 20 ml washing buffer and finally equilibrated for 5 min in 10 ml detection buffer. Membranes were placed with DNA side facing up on a development folder and 0,1 ml CSFD was applied onto the membranes. Samples were incubated for 5 min at RT. After that the membranes were exposed for 10 min to the Fusion FX7 imager.

Sample preparation

Genomic DNA from positive clones, verified by RT-PCR, was isolated as described above. 100 µg gDNA of each sample was cut overnight at 37°C using 300 units Hind*III* in a total reaction volume 400 µl. Digested samples were concentrated using alcohol precipitation. The complete sample was then mixed with 40 µl 3 M NaOAc and vortexed. 440 µl of ice-cold 100% EtOH was added, mixed by vortexing and placed in the -80°C freezer for 1 hour. Tubes were centrifuged for 15 min at 13000 rpm and RT

supernatant was removed. 300 µl 70% EtOH was added, tubes were several times inverted and centrifuged for 5 min at 13000 rpm and RT. Supernatant was removed and pellets were left to air-dry. Samples were than dissolved in 50 µl 1xTE buffer. 20 µg gDNA of each sample was separated using agarose gel electrophoresis on 7 mm 0,7% agarose gel (1xTAE) at 60 V, 8 hours.

Southern blot onto nylon membrane with high-salt buffer

Denaturation: Agarose gel was rinsed in dH_2O, placed in a clean glass dish containing ~10 gel volumes denaturation solution and shaken for 20 min at RT. This step was repeated with a new portion of denaturation solution.

Neutralization: The gel was rinsed in dH_2O and ~10 gel volumes of neutralization solution were added. Samples were then incubated for 20 min while shaking at RT. This step was performed twice with a new portion of neutralization solution.

Equilibration: The neutralization solution was poured off and the gel was equilibrated for 10 min in 20x SSC (gently shaking).

Transfer: A plastic container was filled up with 20x SSC. A sponge, soaked with 20x SSC was placed in the middle of container. The sponge had to be approximately half-submerged in 20x SSC. Then three pieces of Whatman 3MM paper in the same size as the sponge were wetted in 20x SSC and placed on the sponge. Then the gel was placed upside down on Whatman papers and rolled by a plastic pipette to squeeze out air bubbles. Four pieces of parafilm were arranged over the gel edges to prevent buffer from short-circuiting. A piece of nylon membrane just as large to cover the exposed gel surface was poured in dH_2O and incubated for 5 min. The membrane was placed on the gel and wetted with 20x SSC. Five pieces of dry Whatman paper (in the same size as the membrane) were placed on the top of membrane. Finally, paper towels in the same size as the membrane were stacked up on the top of Whatman papers. A glass plate was placed on the top of the “sandwich” and weightened with 200 g additional weight. Finally the transfer was performed O/N (overnight).

Immobilization: on the next day, the membrane was taken out of the sandwich and rinsed in 2x SSC. Then it was placed on a sheet of Whatman paper and allowed to dry. Dried membrane was placed between two sheets of Whatman paper and baked for 30 min at 120°C.

Hybridization: The membrane was covered in 10 ml DIG Easy Hyb buffer pre-warmed to 37°C. Prehybridization was performed for 60 minutes at 37°C, while gently shaking

the samples. DIG-labeled DNA probe was boiled for 5 min and rapidly cooled on ice. The probe was diluted in 5 ml preheated DIG Easy Hyb buffer to a final concentration of 30 ng/ml. The prehybridization solution was removed and membrane was placed into sealed plastic bag and covered with probe mixture. Hybridization was performed O/N at 42°C with gentle shaking.

Stringency washes: The next day, the membrane was washed 2 x 5 min at 42°C with 100 ml 2x SSC/0,1% SDS (prewarmed to 42°C), under constant shaking. Then the membrane was washed 2x15 min at 60°C with 100 ml 0,5x SSC/0,1% SDS (prewarmed to 60°C) under constant shaking.

Immunological detection (all incubation were performed at 37°C with shaking): After stringency washes, the membrane was rinsed for 5 min in 100 ml washing buffer. Then the washing buffer was removed and membrane was incubated for 2 hours in 100 ml blocking solution. After incubation, the blocking solution was removed and membrane was for 60 min incubated in 20 ml antibody solution (1:5000). Samples were 2 x 15 min washed in 100 ml washing buffer and equilibrated for 5 min in 20 ml detection buffer. The membrane was then placed into a plastic bag with DNA side facing up and wetted with 1 ml CSPD ready-to-use. Samples were incubated for 15 min at 37°C. The membrane was placed into development folder and exposed for 20 min to Fusion FX7 imager.

2.2.10 hGPR34 cloning

We obtained hGPR34 gene sequence in pFLAG-CMV^{TM}-3 expression vector (Sigma) cloned in-frame with N-terminal FLAG-tag. We had to re-clone the pFLAG-hGPR34 sequence into pcDNA5/TO vector (Invitrogen) to get the pFLAG-hGPR34 under the control of tetracycline inducible promoter. The pFLAG-hGPR34-pcDNA5/TO vector was used as an initial construct for Jump-In TI Gateway^{TM} cloning as well as for Meganucluase TI and directly for conventional transfection approach.

pFLAG-hGPR34 DNA-fragment was isolated from hGPR34/pFLAG-CMV-3 using *SnaBI* and *XbaI* and cloned into pcDNA5/TO pre-cutted with *EcoR*V and *Xba*I.

Conventional cloning

Fragment restriction:

10 µg of hGPR34 in pFLAG-CMV-3 construct DNA was mixed with 2 µl NEBuffer 4, 6 µl dH_2O, 0,2 µl BSA, 1 µl *SnaB*I and 1 µl *Xba*I. Samples were incubated O/N at 37°C.

Vector restriction:

10 µg of pcDNA5/TO vector was mixed with 2 µl NEBuffer 2, 6 µl dH_2O, 0,2 µl BSA, 1 µl *EcoR*V and 1 µl *Xba*I. Samples were incubated O/N at 37°C. After incubation the sample was treated with 1 µl SAP and incubated another 30 min. SAP was then heat-inactivated for 15 min at 65°C.

Both samples were run on agarose gel (1% agarose in TAE buffer), both fragments were isolated and purified using MinElute Gel Extraction Kit (Qiagen) as described previously on page 42.

Ligation:

50 ng of restricted pcDNA5/TO and 250 ng of pFLAG-hGPR34 fragment were diluted in 1x DNA dilution buffer to total volume 10 µl. Then 10 µl of 2x DNA ligase buffer and 1 µl of DNA ligase was added and samples were incubated overnight at RT.

The next day, 4 µl of the ligated samples was used to transform One Shot® Mach1^{TM} – T1^{R} Chemically Competent *E. coli* as described previously on page 38.

MultiSite Gateway cloning

As the MultiSite gateway vectors do not contain any promoter sequence to drive the GOI transcription and we wanted our expression to be tetracycline inducible, we had to clone both pCMV/TO promoter as well as pFLAG-hGPR34. Moreover we had to include a polyA tail sequence as well. Due to the large size of the whole promoter-pFLAGhGPR34-pA sequence, we decided to clone these as 2 fragments using MultiSite Gateway technology.

Firstly, we had to designe site-specific primers to attach our fragments with specific *att*B sites. Then we perfomed a PCR reaction with these specific primers. As a template DNA served pFLAG-hGPR34 in pcDNA5/TO linearized with *SnaB*I for the reaction with insert and pcDNA5/TO linearized with *BamH*I for the promoter fragment propagation.

„Tab 6“: Reaction mixture for creating of Gateway fragments.

Component	Per reaction
Template DNA 10 ng/µl	2 µl
10x PCR buffer 15 mM $MgCl_2$	5 µl
25 mM $MgCl_2$	1,5 µl
10 mM dNTP mix	1 µl
10 µM sense primer	1 µl
10 µM antisense primer	1 µl
Taq DNA Polymerase (5U/µl)	0,4 µl
PCR H_2O	38,1 µl
Total reaction volume	50 µl

„Tab 7”: PCR setup for Gateway fragments creation. Products from the PCR reaction were analysed using agarose gel electrophoresis and purified using MinElute Gel Extraction Kit as previously described.

PCR setup	Temperature	Time	
First denaturation	94°C	4 min	
Denaturation	94°C	30 sec	35x
Annealing	60°C	30 sec	
Elongation	72°C	60 sec	
End elongation	72°C	10 min	
Cooling	4°C		

Performing of BP recombination reaction (Creating Entry Vectors):

All components were combined in one vial, briefly vortexed and incubated O/N at 25°C. 1 µl of proteinase K was added to stop the reaction and incubated for 10 min at 37°C. 2 µl of both entry vectors obtained by BP reaction were used to transform One Shot® Mach1™ – T1^R Chemically Competent *E. coli* cells. Small overnight cultures were prepared and plasmids were isolated using QIAprep Spin Miniprep kit (Qiagen) as described previously on page 39.

„Tab 8“: Reaction mixture for BP recombination.

BP recombination reaction	Vol (µl)
attB1-pCMV/TO-attB5r fragment DNA 25 ng/µl	1
pDONR 221 P1-P5r vector DNA 50 ng/µl	1,5
1x TE buffer pH=8	5,5
BP Clonase II enzyme mix	2

BP recombination reaction 2nd fragment	Vol (µl)
attB5-pFLAG-hGPR34-BGH-pA-attB2 fragment DNA 45 ng/µl	1,2
pDONR 221 P5-P2 vector DNA 50 ng/µl	1,5
1x TE buffer pH=8	5,3
BP Clonase II enzyme mix	2

Performing of LR recombination reaction (Creating expression construct)

All components were mixed into vial, briefly vortexed and incubated at 25°C, O/N.

A positive control Control was included to monitor the LR recombination between pENTR™ L1-pLac-*lac*Zα-R5, pENTR™ L5-pLac-Spec-L2 and pJTI R4 DEST vector.

„Tab 9”: Reaction mixture for LR recombination reaction.

LR recombination reaction	Vol (µl)
pCMV/TO in pDONR 221 P1-P5r 10,1 ng/µl	2,2
pFLAG-hGPR34-BGH pA in pDONR 221 P5-P2 12,8 ng/µl	2,14
pJTI R4 DEST vector 50 ng/µl	1,48
1x TE buffer pH=8	2,18
LR Clonase™ II enzyme mix (Invitrogen)	2

<u>Cloning into Meganuclease integration matrix</u>

As the pcGPS-C.CHO.MCS integration matrix contains its own pCMV promoter, but we wanted the hGPR34 expression to be inducible, we decided to re-clone the pFLAG-hGPR34 fragment from pcDNA5/TO with SnaBI, which specifically cuts in the pCMV promoter and create a so-called fusion promoter with part of the promoter in the integration matrix and part of the promoter with tetracycline operator sequence. We mixed 10 µg of pFLAG-hGPR34 in pcDNA5/TO with 6 µl dH_2O, 2 µl NEBuffer 4, 0,2 µl BSA, 1 µl *Sma*I and incubated O/N at 25°C. Then 1 µl of *SnaB*I restriction endonuclease was added and incubated at 37°C, O/N. 10 µg of integration matrix was

mixed with 6 µl dH_2O, 2 µl NEBuffer 4, 0,2 µl BSA, 1 µl *SnaB*I, 1 µl EcoRV and incubated O/N at 37°C. Integration matrix was treated with SAP and incubated for another 30 min at 37°C. Fragments were analyzed using agarose gel electrophoresis and isolated using MinElute Gel Extraction Kit as previously described. Fragments were ligated with the vector using Rapid DNA Ligation Kit. The insert-to-vector ratio was 1:5 (50:250 ng) and O/N incubation was performed at RT. 4 µl of ligated samples was used to transform ElectroMAX™ DH5α-E™ Competent Cells. 10 colonies were picked to prepare small overnight cultures with subsequent plasmids isolation. To verify the right orientation of the inserted fragment, 10 µg of each sample was cut using *Cla*I and analysed using agarose gel electrophoresis.

2.2.11 Cells retargeting

Positive and negative controls were included in all performed retargeting experiments.

pFLAG-hGPR34 in pcDNA5/TO and control vector pcDNA5/TO/LacZ expressing β-galactosidase were both linearized with *Nru*I (Buffer O+, 37°C, O/N). Early passage CHO#35 T-Rex cells were seeded one day prior the experiment to be ~80% confluent on the day of the experiment (1.10^6 cells for Ø 14,5 cm culture Petri dish). One hour prior to the transfection, culture medium was removed, and cells were washed once with 1x PBS and fresh transfection medium was added (without blasticidin). 33 µg of the GOI containing vector or LacZ control vector was diluted in 4,125 ml of Opti-MEM®. 165 µl of Lipofectamine™ 2000 reagent was gently mixed, added into 4,125 ml Opti-MEM® and incubated for 5 min at RT. After incubation, diluted DNA was mixed with diluted lipofectamine and incubated for another 20 min at RT. The whole mixture was added to plated cells and incubated for 4-6 hours at 37°C in humidified atmosphere of 5 % CO_2. After incubation, an old medium was removed, cells were once washed with 1x PBS and fresh transfection medium was added. The next day, cells were switched to the selection medium (including blasticidin and hygromycin).

Jump-In Retargeting

One day prior to the experiment early passage CHO#35 T-Rex Zeo platform cells were seeded to be ~80% confluent at the day of the experiment (1.10^6 cells for Ø 14,5 cm culture Petri dish). One hour prior to the transfection culture medium was removed, and cells were once washed with 1x PBS and replaced with transfection medium (omits blasticidin and hygromycin). 11,5 µg of the expression construct and 11,5 µg of the pJTI R4 Integrase expressing vector were combined in a total volume of 4,125 ml Opti-MEM®. 165 µl of Lipofectamine™ 2000 reagent was gently mixed, added to 4,125 ml Opti-MEM® and incubated 5 min, RT. After incubation, diluted DNA mixed was with

diluted lipofectamine and incubated for another 20 min at RT. The whole mixture was added to plated cells and incubated for 4-6 hours at 37°C in humidified atmosphere of 5 % CO_2. After incubation, an old medium was removed, cells were once washed with 1x PBS and poured with fresh portion of transfection medium. The next day, cells were switched to the selection medium (including blasticidin and zeocin, without hygromycin).

Meganuclease Retargeting

One day prior to the experiment, early passage CHO#35 T-Rex cells were seeded in Ø 10 cm culture Petri dish ($2x10^5$ cells per dish). One hour prior to the transfection culture medium was removed, cells were once washed with 1x PBS and replaced with transfection medium (without blasticidin). 1 µg of the meganuclease expressing vector and 2 µg of the GOI containing integration matrix or LacZ expressing integration matrix were diluted in 275 µl of Opti-MEM®. 25 µl of PolyFect™ reagent was added, briefly vortexed and incubated for 10 min at RT. Transfection mixture was mixed with 700 µl of transfection medium and added to the plated cells. Cells were incubated for 3 days at 37°C and atmosphere of 5% CO_2. 3 day post-transfection cells were switched to the selection medium (6 µg/ml blasticidine, 600 µg/ml hygromycin). 14 days post-transfection 5 µg/ml 6-thioguanine was added to the selection medium.

2.2.12 FACS (Fluorescence-Activated Cell Sorting) analysis

Antibodies solutions were prepared immediately before use by dilution in ice-cold PBS.

Final AB concentrations:

monoclonal anti-flag M2 produced in mouse 1,5 µg/100µl
mouse anti-IgG_1 1 µg/100 µl
FITC labeled goat anti-mouse 2 µg/100 µl

Plated cells were washed once with 1 ml 1xPBS, detached with 1 ml EDTA/PBS solution (incubation for max. 1 min at 37°C, cells detached by pipetting) and transferred into FACS tubes. 1 ml of ice-cold PBS was added to the cells and samples were centrifuged for 10 min at 1000 rpm and 4°C. Supernatant was then poured off and pellets resuspended in 100 µl AB1 solution. Samples were briefly vortexed and incubated for 45 min at 4°C. 500 µl of ice-cold PBS was added to each sample and tubes were centrifuged for 10 min at 1000 rpm and 4°C. The supernatant was discarded and pellets were resupended in 100 µl of AB2 solution, vortexed and incubated for 45 min at 4°C in the dark. Tubes were filled up with 500 µl ice-cold PBS

and centrifuged for 10 min at 1000 rpm and 4°C. Finally the supernatant was discarded and the cell pellets were resuspended in 500 µl ice-cold PBS.

2.2.13 X-Gal staining

For all retargeting experiments were included positive controls with the appropriate *Lac*Z gene containing plasmid which subsequently leads to β-galactosidase expression after successful integration.

X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) is an organic colorless compound used to differentiate positive recombinants from non-recombinants in a blue-white screening test. After X-gal addition it is recognized by β-galactosidase and cleaved to produce galactose and 5-bromo-4-chloro-3-hydroxyindole which dimerizes and is oxidized with athmospheric oxygen and $K_4Fe(CN)_6/K_3Fe(CN)_6$ equimolar mixture into 5,5'-dibromo-4,4'-dichloro-indigo – a water insoluble intensively blue compound (Kiernan, 2007).

Medium from tested cells (seeded in Ø 10 cm culture Petri dish) was removed and 10 ml of fixing solution was added. Cells were incubated 10 min, RT. Fixing solution was then removed and cell were washed 3 times with 10 ml 1xPBS. During the second washing step PBS was left on the cells for 10 min. 5 ml of staining solution was added to cells and cells were incubated for 3 hours at 37°C. The percentage of positive cells was detected via microscopy.

2.2.14 Tested compounds and their dilution

All tested compounds were purchased from Sigma-Aldrich. All compounds are further referred to by their catalogue number („Tab. 10")

„Tab. 10": Tested compounds and their reference numbers.

Compound name	Cat. Nr.
L-α-Lysophosphatidylinositol sodium salt from soybean	62966
3-sn-Phosphatidylethanolamine from bovine brain	P7693
Arachidonic acid	A3555
1,2-Diacyl-sn-glycero-3-phospho-L-serine	P6641
1-palmitoyl-sn-glycero-3-phospho-l-serine	51581
L-α-Lysophosphatidylcholine from bovine brain	L1381
L-α-Lysophosphatidylcholine from egg yolk	L4129
Sphingosylphosphorylcholine	S4257
Sphingosine 1-phosphate	S9666

Stock solutions were prepared by dilution in a mixture of chloroform:methanol (1:1) to the final compound concentration 20 mM. Working solutions were prepared by further dilution in methanol to a final concentration of 2 mM.

2.2.15 cAMP HTRF assay

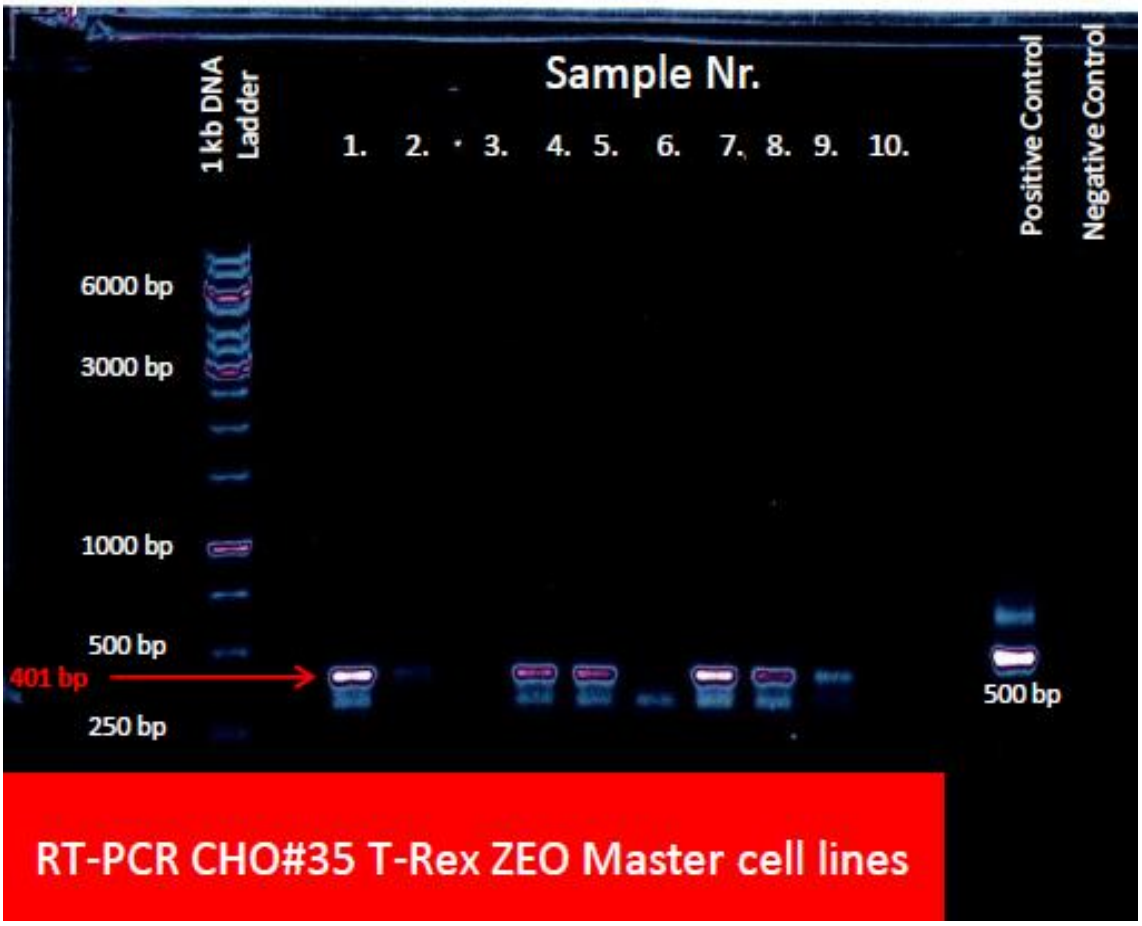
One day prior to the experiment cells were seeded at 6000 cells per well into white 384-well plates and centrifuged for 5min at 800 rpm. At the day of the experiment, I prepared a compound plate by pipetting 20 of µl coumpound (100x concentrated) into a clear 384-well plate. Forskolin or calcitonin plates were prepared by dilution in PBS/F-68 to a final concentration 2,5 µM forskolin and 1 µM calcitonin. 384-well clear plates were filled up with 50 µl of the forskolin or calcitonin solution.

Medium from plated cells was removed and 0,5 µl of each compound was transferred from the compound plate to the well with 50 µl of buffer from stimulation plate with either forskolin or calcitonin, mixed by pipetting and 15 µl of the compound mixture was then transferred to the cells. Cells and compounds were incubated for 1 hour at 37°C. Then 5 µl of conjugate and lysis buffer were added (5 µl cAMP-D2 and 5 µl anti-cryptate). Samples were briefly centrifuged (~1000 rpm) and incubated for 1 hour at 20°C. Samples were measured using the EnVision Microplate reader using an excitation wavelength 320 nm and two emission wavelengths 620 and 665 nm.

2.3 Results

2.3.1 RT-PCR

A RT-PCR reaction was performed to verify the integration of platform vector into the transcriptionally active site in the genome of targeted cells. RNA samples from 10 different clones were isolated to verify generation of novel CHO#35 T-Rex Zeo platform cell lines. Positive clones were selected by using selection medium containing 6 µg/ml blasticidin and 600 µg/ml hygromycin. Two-weeks post-transfection, we isolated whole cell RNA and performed RT-PCR with non-specific Oligo(dT)$_{12\text{-}18}$ in the reverse transcriptase reaction and specific self-designed primers *att*P (+) and Zeo (-) in the amplification reaction covering the region of the *att*P site naturally occuring in genome and zeocin resistance gene. A succesful reaction would lead to PCR-products with a length of 401 bp. Samples 1.,4.,5.,7. and 8. looked positive and were kept for future experiments and used in a subsequent Southern blot analysis.



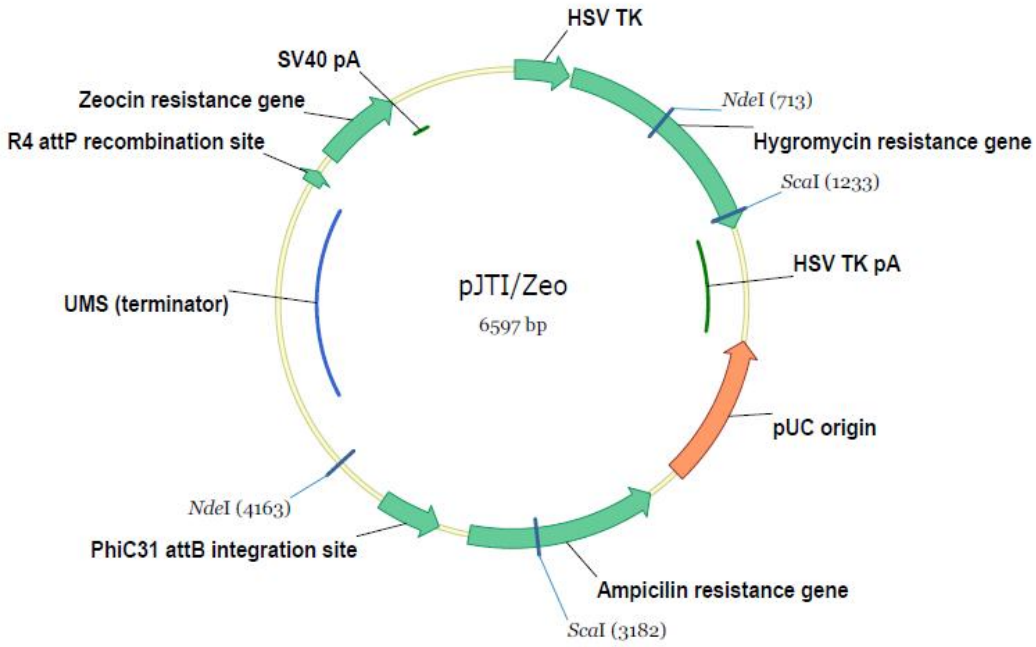
„Pic. 12": RT-PCR reaction with subsequent analysis using agarose gel electrophoresis (1x agarose, 1x TAE buffer). Succesful integration into transcriptionally active site leads to the PCR product with a length of 401 bp. Positive control stands for RNA sample included in the SuperScript™ First Strand Synthesis System (Invitrogen) with control Primers A and B leading

to a product of the lenght of 500 bp. Negative control stands for NO RT reactions as no reverse transcriptase was added into the sample. β-actin primers were used as a internal control leading to a band running at the size of 294 bp. (Picture was acquired with Fusion FX7 imager, Vilber Lourmat)

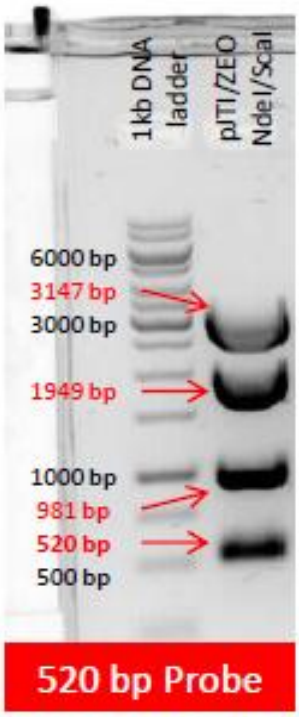
2.3.2 Southern blot

Southern blot analysis was used to determine the number of integration sites in the platform cells genome. Samples 1.,4.,5.,7. and 8. previously found out as a positive in RT-PCR reaction were tested. We isolated genomic DNA, cut it with *Hind*III and run samples on a 7 mm thick 0,7% agarose gel without staining.

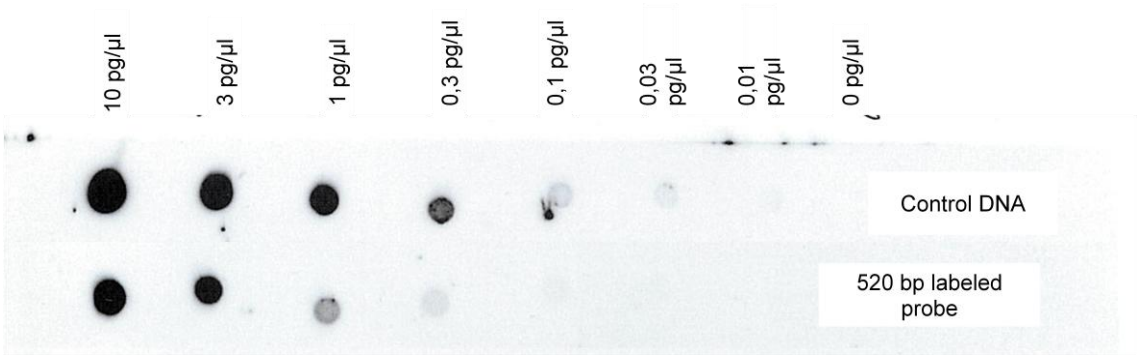
The probe for labeling was isolated from the region of pJTI^{TM}/Zeo platform vector after cutting with *Nde*I and *Sca*I HF. Fragments were run on 1% agarose gel to be separated. A 520 bp fragment was extracted from an agarose gel and labeled by insertion of digoxigenin labeled dUTP using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The Probe labeling efficiency was compared with labeled control DNA so that we obtained an approximate concentration of successfully labeled probe.



„Pic. 13": Scheme of the pJTI^{TM}/Zeo platform vector. Fragment between *Nde*I (713 bp) and *Sca*I (1233 bp) was isolated and used for subsequent labeling reaction as Southern blot probe (Self-made picture, Vector NTI 11).

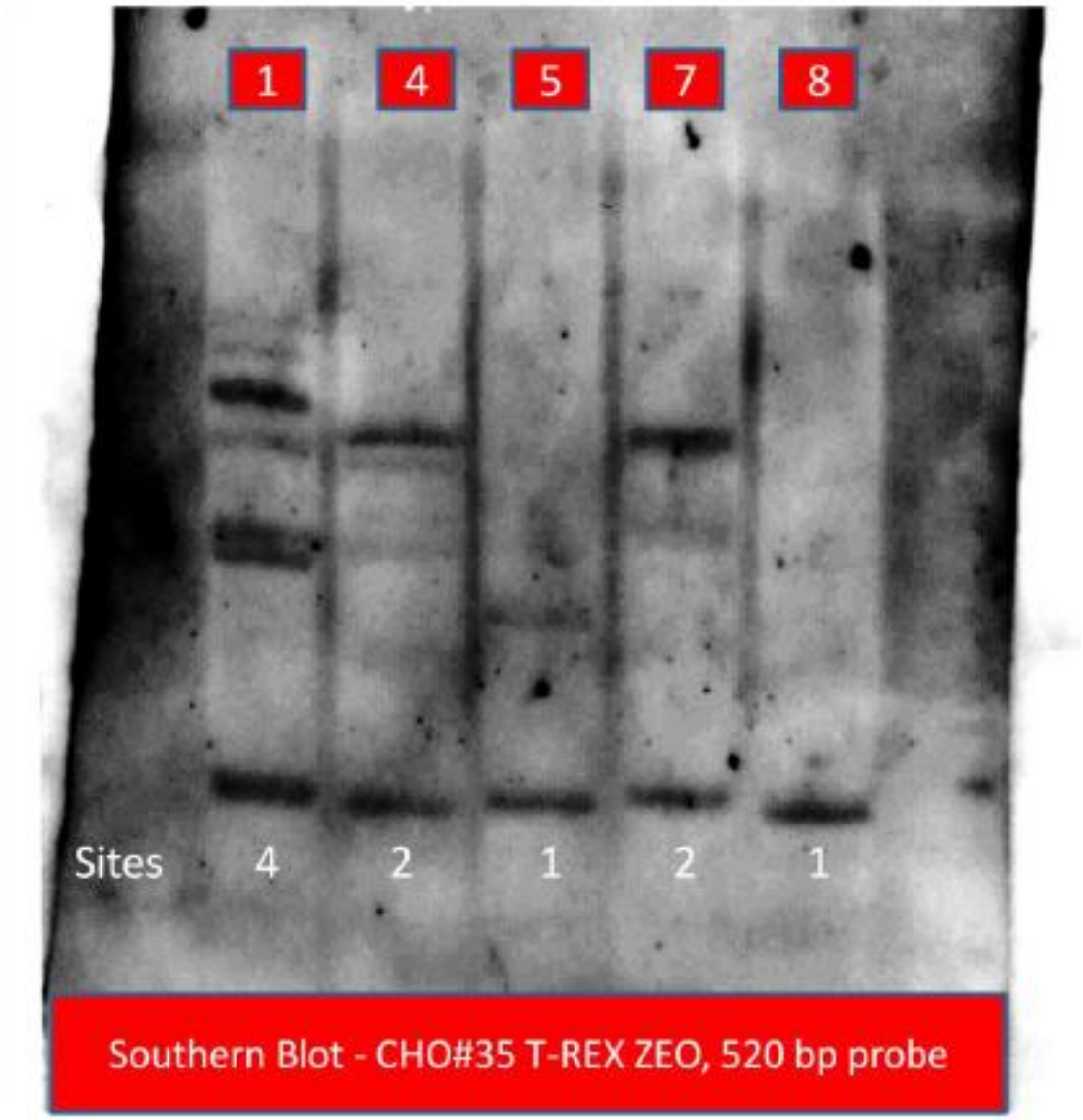


„Pic. 14": Agarose gel electrophoresis of the pJTI/Zeo cut with *Nde*I and *Sca*I HF restriction endonucleases. Red arrows are pointing on four different fragments obtained from restriction. Fragment of the length of 520 bp was extracted from the gel and used for subsequent labeling reaction. (Picture was acquired with Fusion FX7 imager, Vilber Lourmat)



„Pic. 15": Control reaction for the probe labeling efficiency. Final concentration of labeled probe was determined as ~0,3 ng/µl. (Picture was acquired with Fusion FX7 imager, Vilber Lourmat)

Southern blot transfer was accomplished by a high-salt buffer transfer onto positively charged nylon membrane. DNA was crosslinked onto membrane by baking at 120°C for 30 min. A DIG-dUTP labeled probe was used at a concentration of 30 ng/ml. Incubation with 520 bp probe was overnight at 42°C. Post-hybridization stringency washes were performed at 42°C and subsequently at 60°C. The final antibody dilution was 1:5000 and exposition time was 20 min.

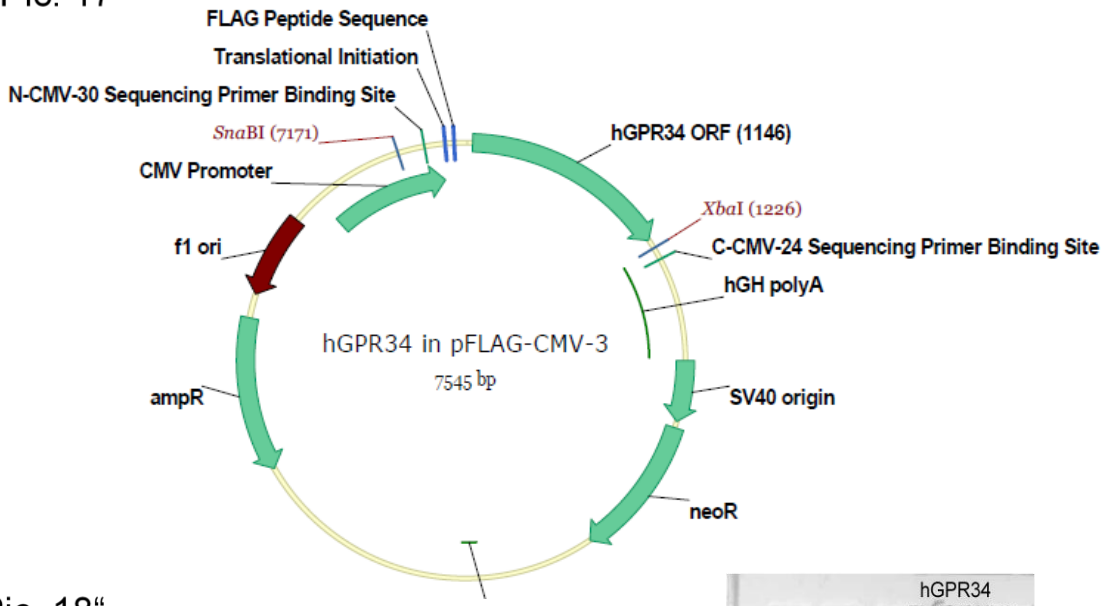


„Pic. 16": Southern blot analysis of the gDNA isolated from established platform cell line CHO#35 T-Rex Zeo. We found different number of integration sites across our samples. For subsequent retargeting experiments we chose samples 1. and 4.; with 4 having 2 integration sites respectively. Additional weak bands may refer to a non-specific bound probes caused by insufficient washing. (Picture was acquired with Fusion FX7 imager, Vilber Lourmat)

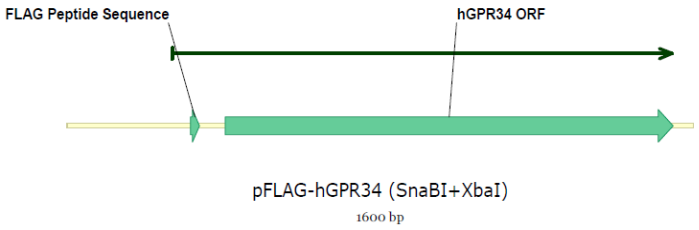
2.3.3 hGPR34 cloning

The pFLAG-hGPR34 fragment was isolated from starting vector pFLAG-CMV3 after RE-digestions using *SnaB*I and *Xba*I. The 1600 bp fragment of interest was isolated *EcoR*V, *Xba*I).

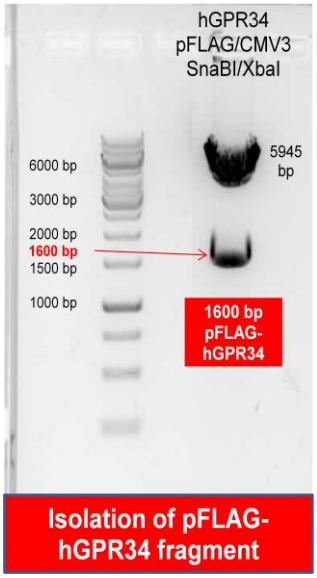
„Pic. 17“



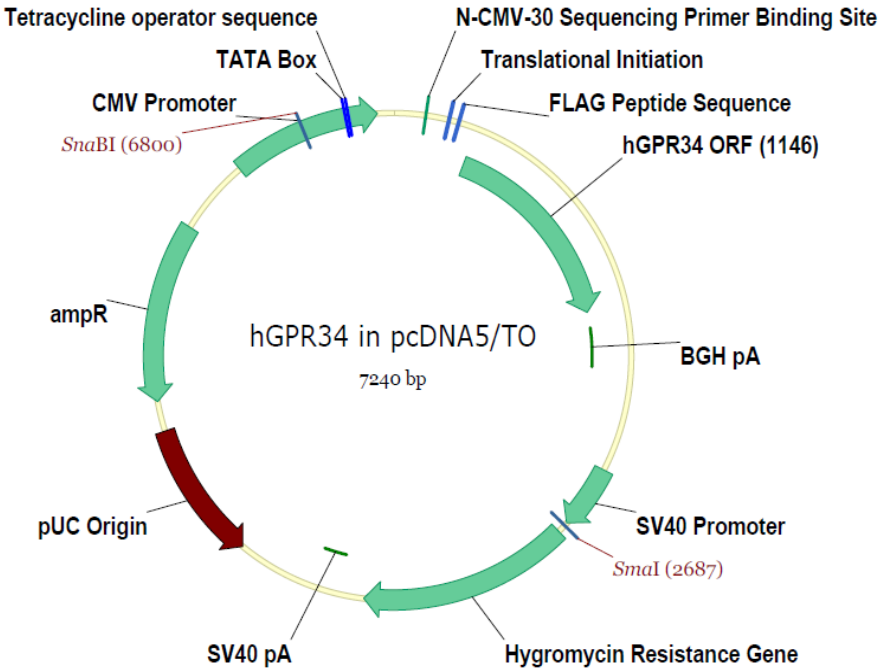
„Pic. 18“



„Pic. 19“



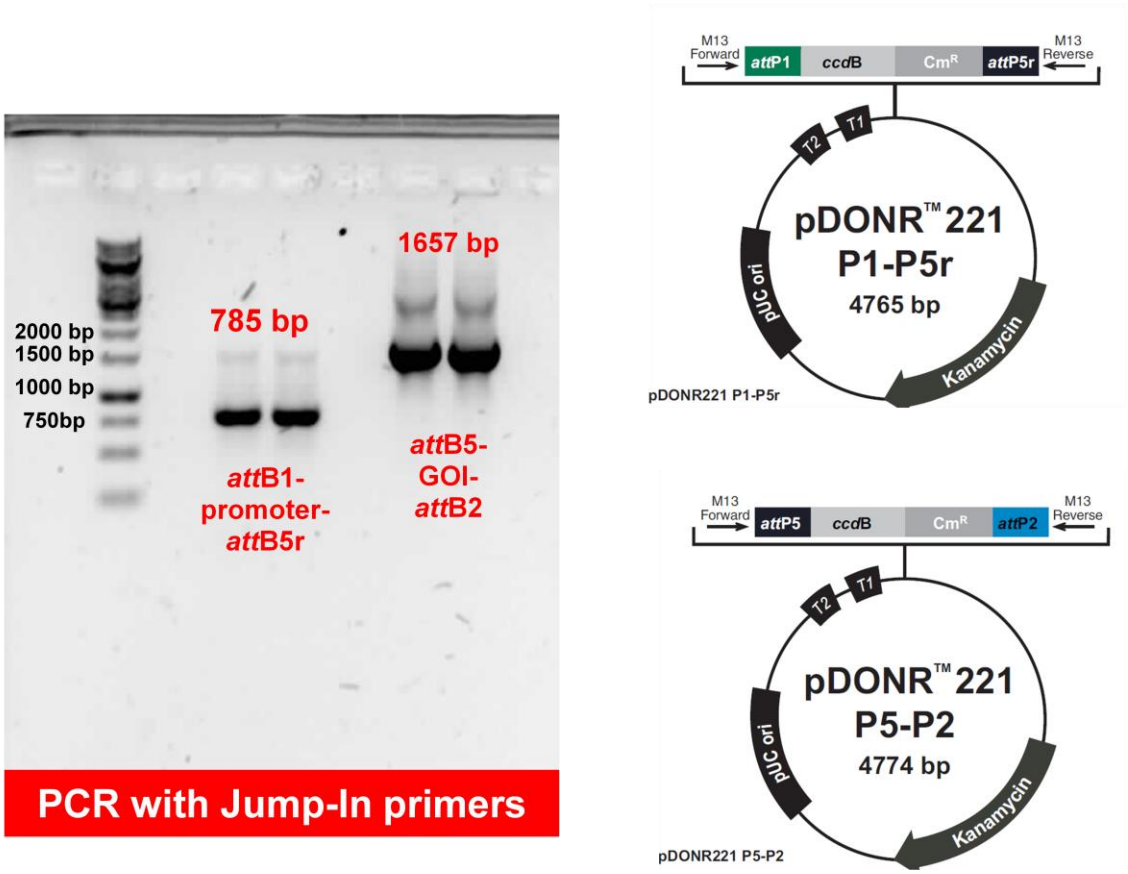
„Pic. 17, 18, 19“: „Pic. 17”: original vector from which the pFLAG-hGPR34 sequence was further cloned. „Pic. 18”: pFLAG/hGPR34 sequence obtained from restriction with *SnaB*I and *Xba*I. „Pic. 19”: pFLAG/hGPR34 sequence after restriction and analysis on agarose gel. This sequence was isolated and further purified using a gel extraction kit as previously described.



„Pic. 20": pFLAG-hGPR34 cloned into pcDNA5/TO vector. This vector served for direct conventional cloning into T-Rex cell lines, and as a template for the Jump-In PCR reaction, as well as for restriction and cloning into Meganuclease integration matrix with marked *SnaB*I and *Sma*I restriction sites.

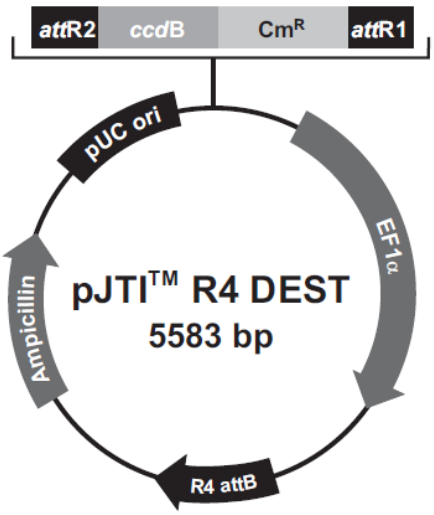
2.3.3.1 Gateway cloning

We performed a PCR reaction with specifically designed primers containing *att* attachment sites. PCR products were analyzed using agarose electrophoresis and cloned into appropriate donor vectors. Both donor vectors were cloned into destination vector pJTI R4 DEST and used for re-targeting into the respective host cells. Using Gateway cloning approach allowed us to obtain ENTRY clones including desired fragments to be cloned into DEST vector in subsequent cloning step to create an expression clone.



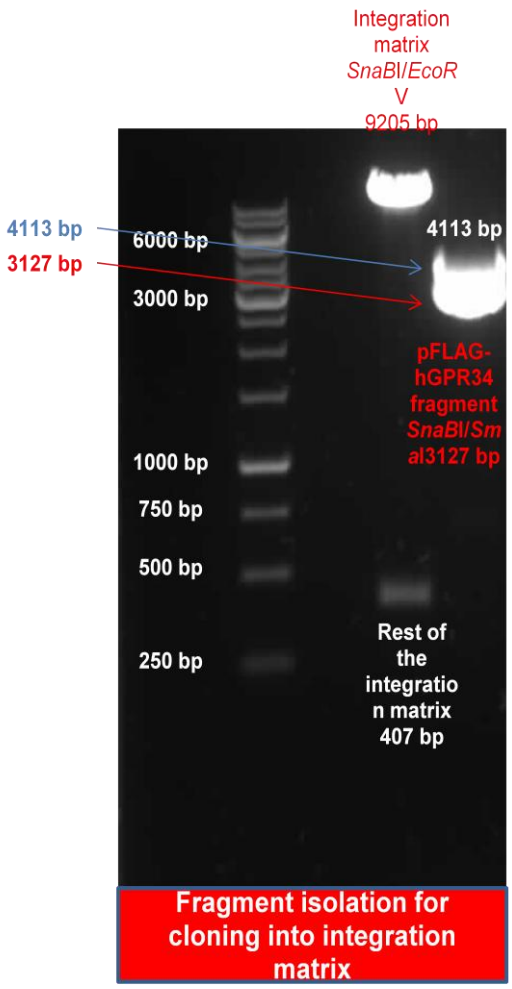
„Pic. 21": Products of the PCR reaction were analysed using agarose gel electrophoresis, isolated and cloned with BP reaction into appropriate pDONR vectors addressing specific *att*P attachment sites. DONR vectors containing the inserted fragment are also called ENTRY clones using using Gateway-Technology nomenclature.

Products of the BP recombination reaction were propagated using One Shot® Mach1™ – T1^{R} Chemically Competent *E. coli* and selected for positive clones using LB agar plates containing kanamycin. Five colonies from each plate were isolated and used for LR recombination reaction together with the pJTI R4 DEST vector.



„Pic. 22": Main features of pJTI R4 DEST vector. Successful selection after LR recombination reaction is guaranteed due to the presence of ampicilin resistance gene as well as *ccd*B gene, which is removed during recombination step. When recombination fails, ENTRY clones do not propagate due to the presence of kanamycin resistance gene as well as empty DEST vector due to the presence of *ccd*B (Source of the picture: Invitrogen).

2.3.3.2 Cloning into integration matrix

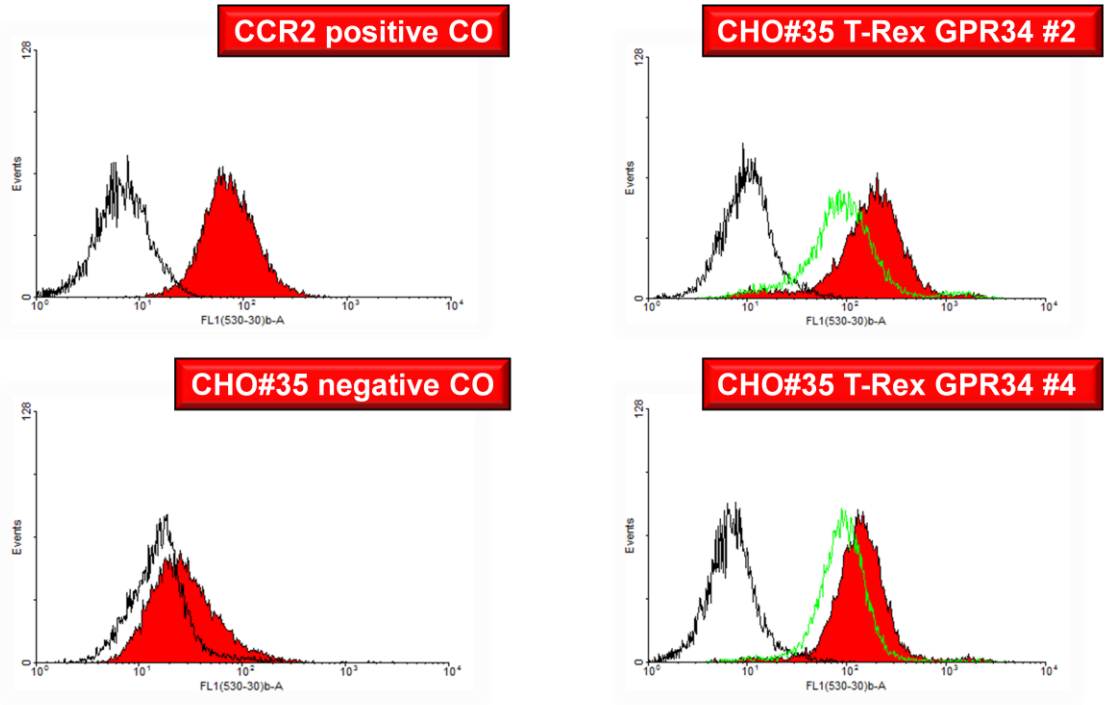


As the integration matrix contains its own promoter and we wanted hGPR34 expression to be inducible, we had to cut the original promoter and create a so-called hybrid promoter with one part belonging to integration matrix and the second part from pcDNA5/TO vector. Therefore pFLAG-hGPR34 sequence was cut out of the pcDNA5/TO vector with *SnaB*I and *Sma*I restriction endonuclease (fragment of the length 3127bp); the integration matrix was pre-cut with *SnaB*I and *EcoR*V (fragment of the length 9205 bp). As these enzymes give blunt ends, we isolated 20 clones and verified them by restriction analysis (Data not shown). Among 20 clones being tested, we obtained only one construct with inserted DNA in right orientation. Right construct was used for re-targeting in meganuclease-driven integration.

„Pic. 23": Isolation of the pFLAG/hGPR34 fragment (3127 bp) with part of the inducible promoter after resctriction with *SnaB*I and *Sma*I.

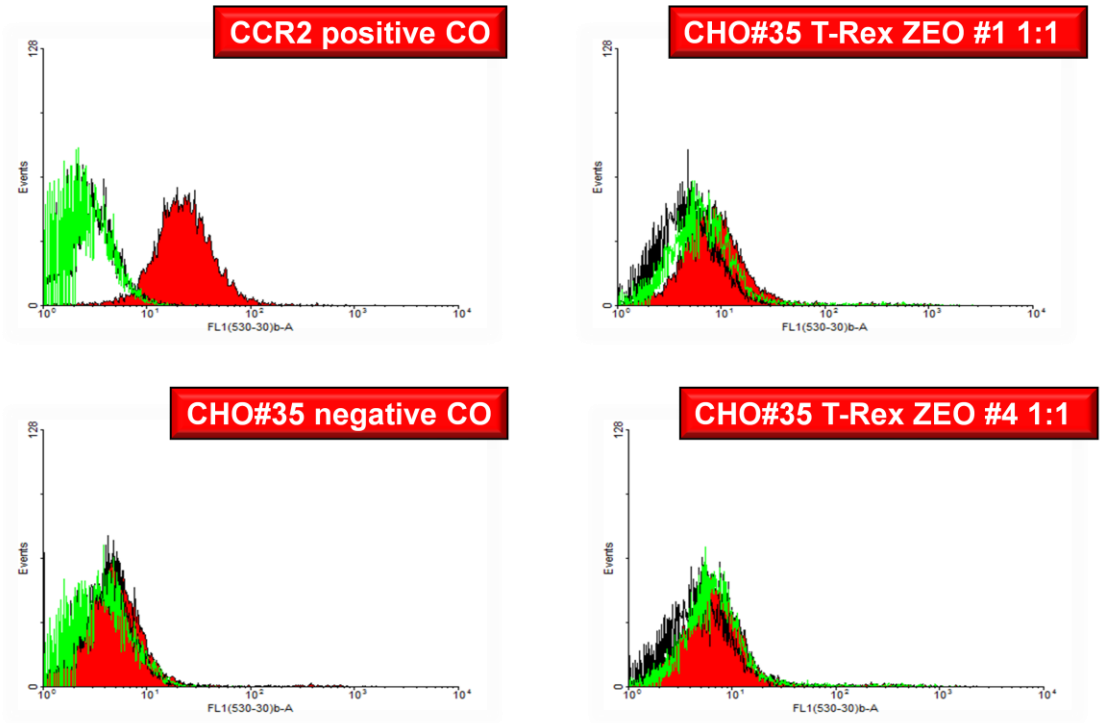
2.3.4 FACS analysis

FACS analysis was used to verify successful surface expression of hGPR34 receptor in the cells. We utilized the presence of Flag-expressed sequence tag. FLAG-tagged CCR2 cells were used as a positive control and as the negative control the parental cell line CHO#35 from which we started the generation of novel cell lines. Primary antibodies were mouse monoclonal anti-flag antibody and mouse anti- IgG_1, secondary antibody was goat FITC-labeled anti-mouse antibody. Mouse anti- IgG_1 antibody was used to determine basal peak.



Black – anti-IgG antibody, negative control; green – uninduced cells with anti-Flag antibody; red – cells induced with 1 µg/ml tetracycline (24 h) with anti-Flag antibody

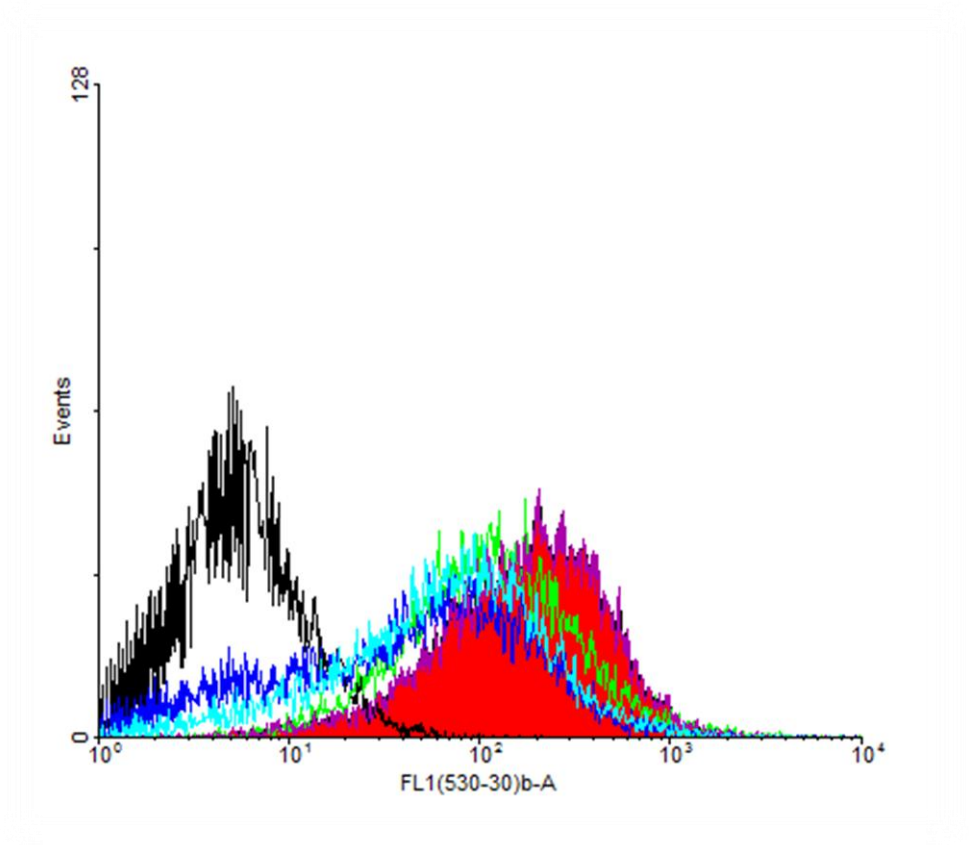
„Pic. 24": Cells obtained by conventional non-directed integration. Left on top CCR2 cells – positive control, left below CHO#35 mother cells – negative control, right shown best gained clones #2 and #4.



Black – anti-IgG antibody, negative control; green – uninduced cells with anti-Flag antibody; red – cells induced with 1 µg/ml tetracycline (24 h) with anti-Flag antibody

„Pic. 25“: Cells obtained with Jump-In Gateway targeted integration Left on top CCR2 cells – positive control, left below CHO#35 mother cells – negative control, right two clones derived from platform cell line CHO#35 T-Rex ZEO #1 and #4.

As we observed a shift of the peak even with uninduced pFLAG/hGPR34-containing cells more closely to induced cells, we decided to be more stringent in the case of meganuclease approach. We started using higher tetracycline concentration to monitor if we could see a shift more towards higher hGPR34 expression. We also tried to transiently transfect our cells with expression vector containing the tetracycline repressor to check if we could see a shift back to the basal peak. As we can observe from peaks where the stable expression was supported by transient expression and the levels of the repressor in the cell were increased, the population does indeed shift back to towards the signal of the parental cells. This could mean that during the course of the cell line generation the stable cell line CHO#35 T-Rex has been loosing its repressor expression abilities.

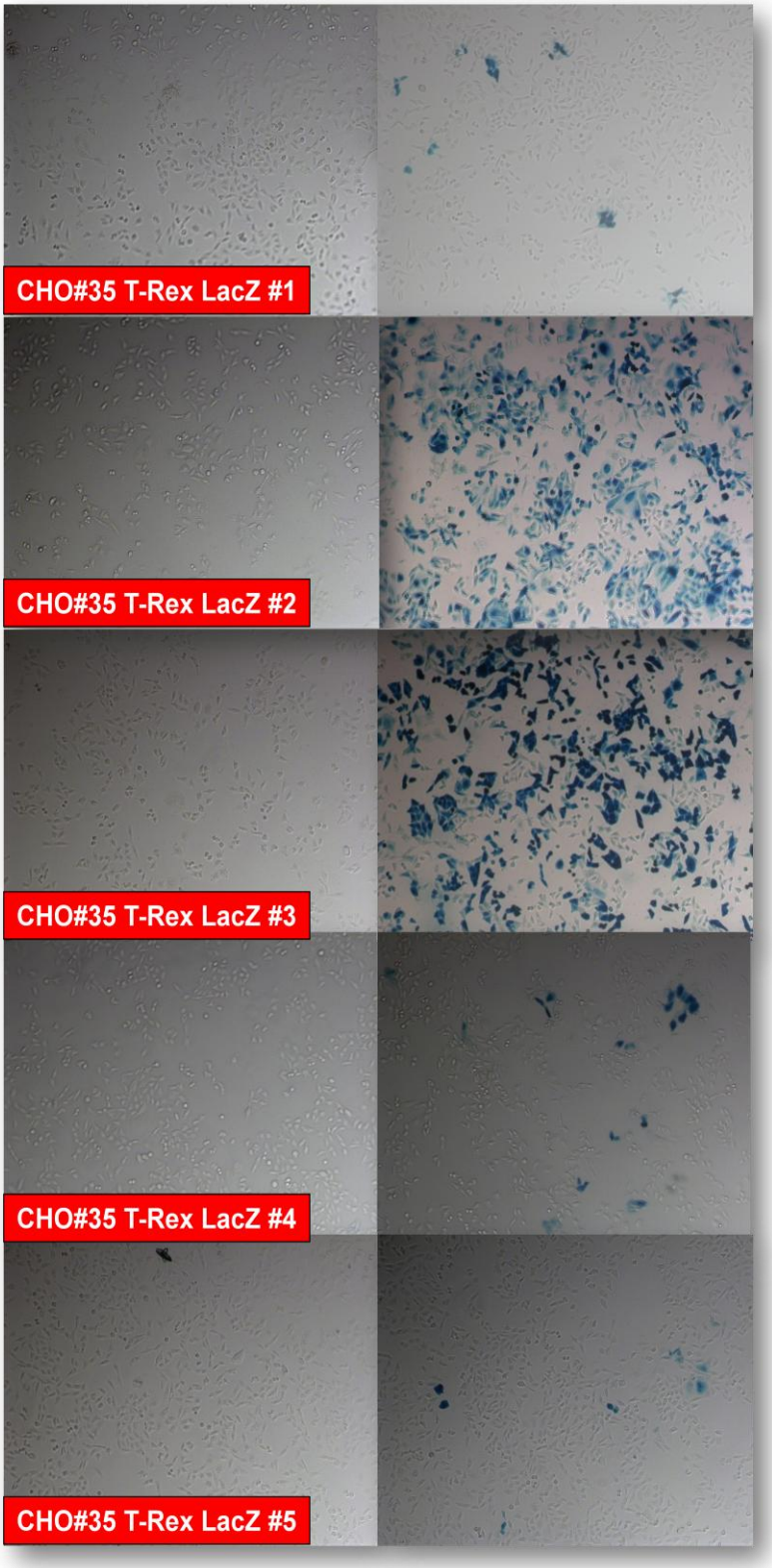


- **red – antiFLAG 1 µg/ml tetracycline**
- **green – no tetracycline**
- **black – mIgG**
- **violet – antiFLAG+ 6 µg/ml tetracycline**
- **cells transiently transfected with TetR, all stained with antiFLAG (dark blue – 18ug/dish TetR; light blue – 10ug/dish TetR)**

„Pic. 26“: Cells obtained from Meganuclease-driven TI approach. As we observed bad repressor properties of isolated populations, we tried to spread our experiment and include a transient transfection of the vector expressing tetracycline repressor. Meaning of all peaks is described in the legend.

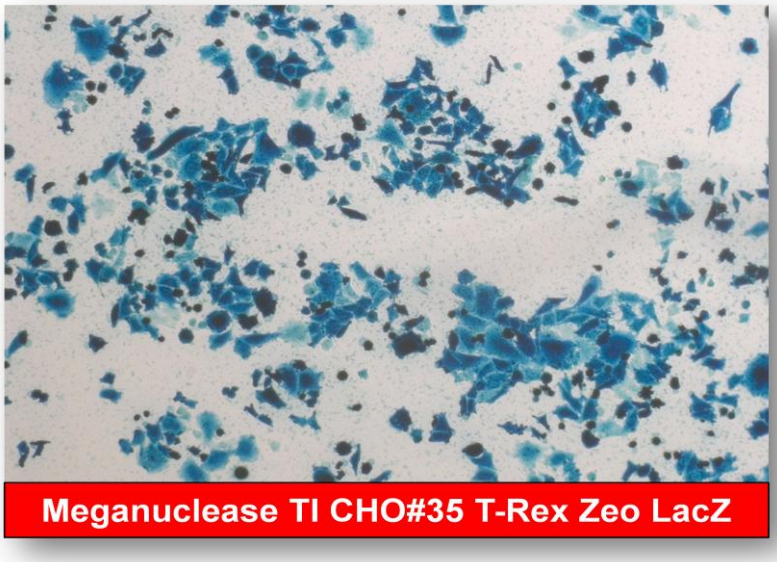
2.3.5 X-Gal staining

X-Gal staining was performed as the control reaction to all transfection experiments when using the control vector containing β-galactosidase gene. Cells were first fixed using glutaraldehyde solution, then stained with X-Gal containing staining solution and incubated for 3 hours at 37°C.



„Pic. 26": Conventional transfection with pcDNA5/TO/LacZ vector. This control vector contains tetracycline inducible promoter pCMV/TO, thus the cells had to be pre-stimulated with culture medium enriched with 1 µg/ml tetracycline 24 hours prior the experiment. Pictures of five different clones picked from transfected pools (left without tetracycline addition, right with tetracycline addition) illustrate how the transfection efficiency differs between population during conventional transfections.

„Pic. 27”: Stable cell line generated with Jump-In targeted integration approach. CHO#35 T-Rex Zeo platform cells were retargeted with expression construct generated by LR catalysed site-specific recombination between pJTI R4 Dest vector and two control vectors L1-pLac-*lac*Zα-R5 and L5-pLac-Spec-L2. As recombination and insertion of these control vectors should lead to immense β-galactosidase expression and no cell turned in blue, thus the Jump-In TI approach did fail in our hands.



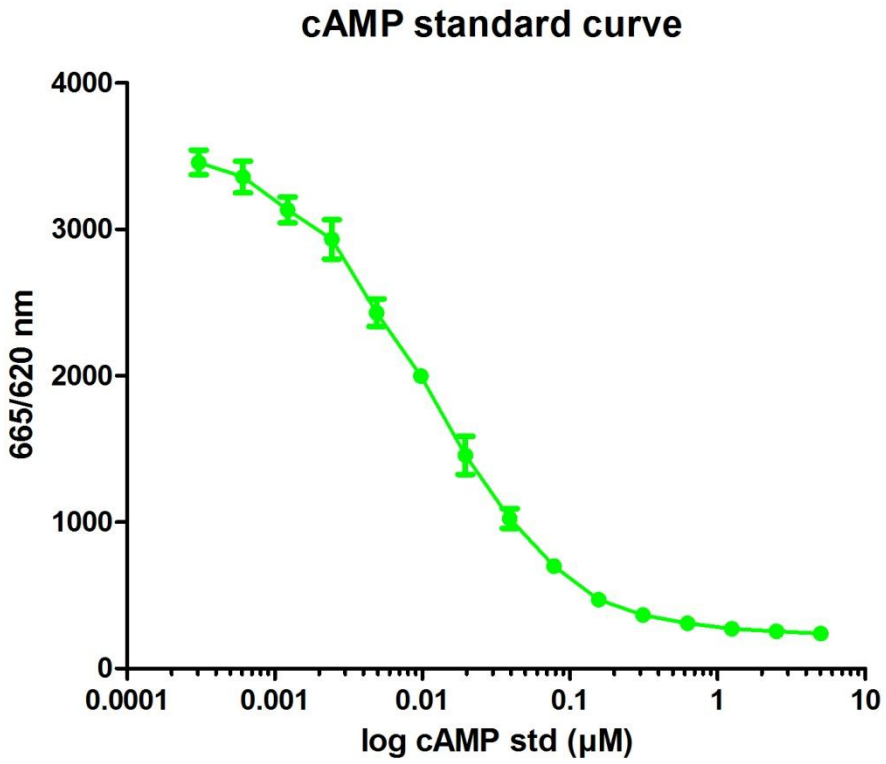
„Pic. 28”: Meganuclease-driven targeted integration with the control integration matrix containing LacZ gene (pcGPS-C.CHO.LacZ). Transfection led to successful homologous recombination and large expression of the β-galactosidase. Due to the smart selection system and rigid integration into familiar position in the genome, we obtained solid cell population without any additional need of sorting or further characterization.

2.3.6 cAMP HTRF assay

Firstly, to determine the final cAMP concentration in the cell, we had to determine a cAMP standard curve. Samples were prepared according to the table below („Tab. 11).

„Tab: 11": Sample dilution for cAMP standard curve arrangement.

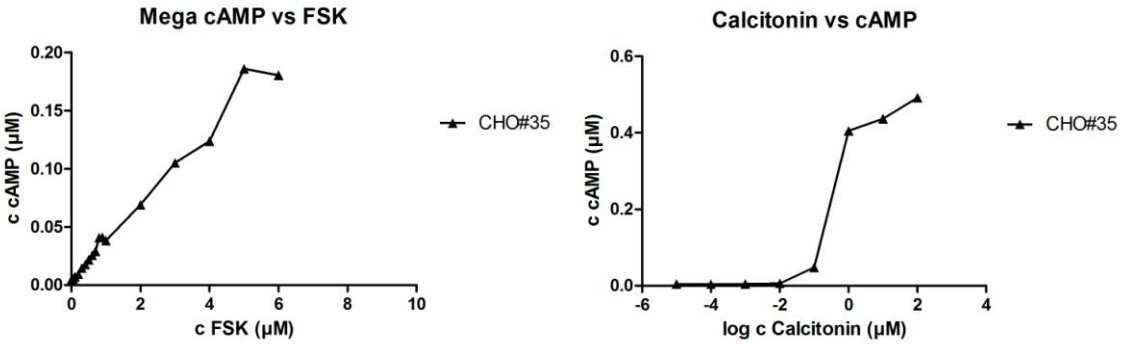
Standard Nr.	Preparation	cAMP working solution (nM)
#1	Reconstituted standard reagent	5000
#2	20 µl #1 + 60 µl buffer	1250
#3	20 µl #2 + 60 µl buffer	312,5
#4	20 µl #3 + 60 µl buffer	78,13
#5	20 µl #4 + 60 µl buffer	19,53
#6	20 µl #5 + 60 µl buffer	4,88
#7	20 µl #6 + 60 µl buffer	1,22
Positive Control	60 µl buffer	0



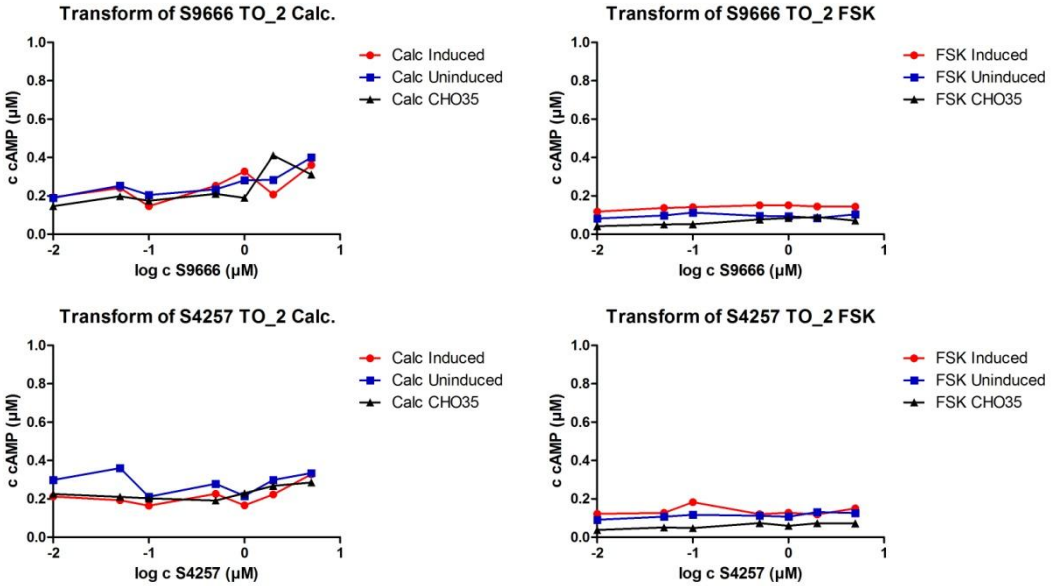
„Pic. 29": cAMP standard curve. X axis represents the concentration of cAMP standard on a logarithmic scale, y axis represents the ratio between signals measured at 665 nm and 620 nm. Data set was obtained from measurement in triplets and analysed using GraphPad Prism 5 as an equation of sigmoidal dose-response.

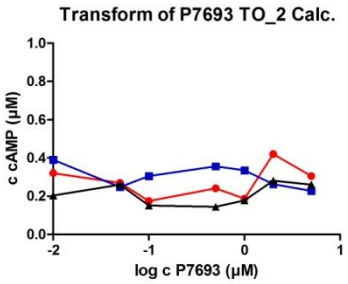
Than we performed the cAMP assay with forskolin and calcitonin both at different concentrations in order to determine the main concentration for further stimulation with

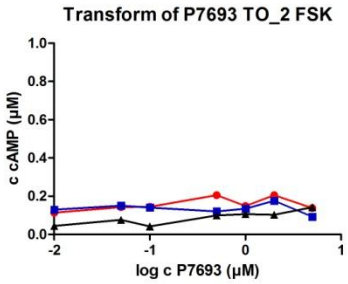
investigated compounds. We determined this concentration to be at 1 µM for forskolin and 100 nM for calcitonin („Pic. 30"). As we obtained very identical results for both clones from conventional cloning (clone #2 and #4) as well as with cell line obtained from Meganuclease approach, I will further present the results for CHO#35 T-Rex clone #2 from conventional cloning only.

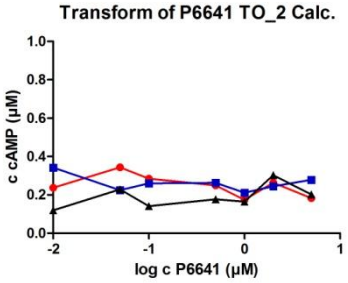


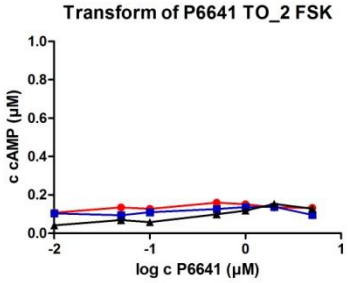
„Pic. 30": Mother cell line CHO#35 (from which CHO#35 T-Rex was derived) was tested in cAMP HTRF assay with stimulation with either forskolin (FSK) or calcitonin to determine the concentration for further stimulation. Cells were stimulated for 1 hour at 37°C and in the atmosphere of 5% CO_2. We decided to use in our experiments 1 µM forskolin and 100 nM calcitonin. X axis represents compound concentration c [µM] (in a logarithmic scale in the case of calcitonin), Y axis represents the real cAMP concentration in the cell lysate calculated from cAMP standard curve using GraphPad Prism 5 as an equation of sigmoidal dose-response. Data set was obtained from measurement in triplets.




Transform of P7693 TO_2 Calc.
c cAMP (µM)
log c P7693 (µM)
Calc Induced
Calc Uninduced
Calc CHO35


Transform of P7693 TO_2 FSK
c cAMP (µM)
log c P7693 (µM)
FSK Induced
FSK Uninduced
FSK CHO35


Transform of P6641 TO_2 Calc.
c cAMP (µM)
log c P6641 (µM)
Calc Induced
Calc Uninduced
Calc CHO35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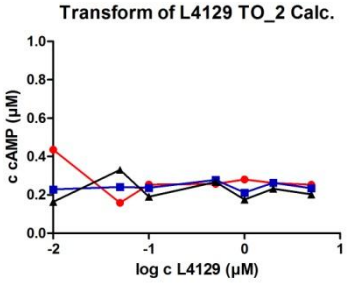
log c P6641 (µM)
FSK Induced
FSK Uninduced
FSK CHO35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log c L4129 (µM)
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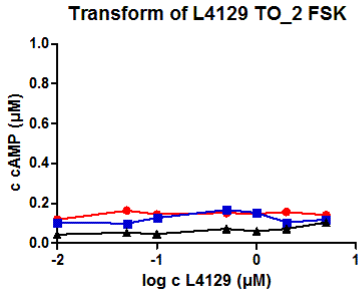
Calc Uninduced
Calc CHO35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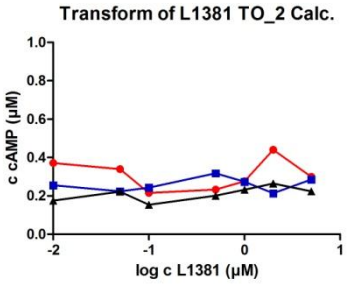
log c L4129 (µM)
FSK Induced
FSK Uninduced
FSK CHO35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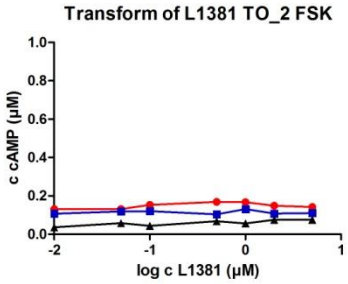
Calc Uninduced
Calc CHO35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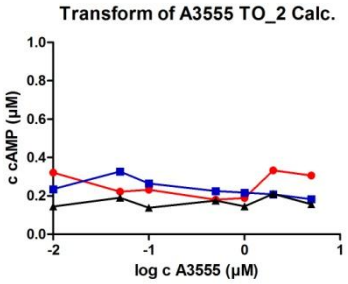
FSK Induced
FSK Uninduced
FSK CHO35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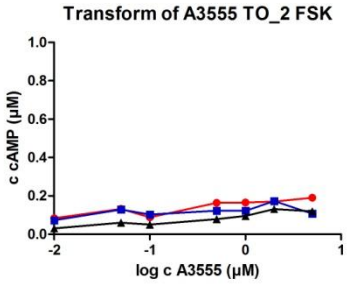
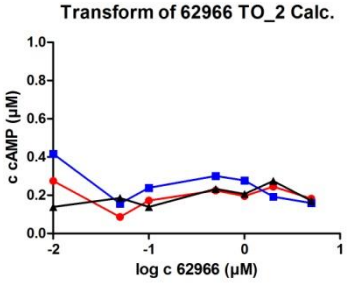
Calc Induced
Calc Uninduced
Calc CHO35


Transform of A3555 TO_2 FSK
c cAMP (µM)
log c A3555 (µM)
FSK Induced
FSK Uninduced
FSK CHO35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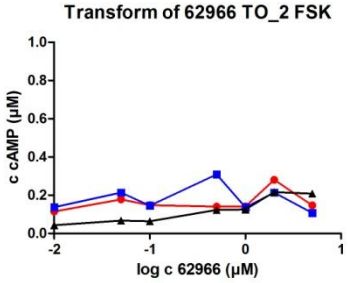
Calc Induced
Calc Uninduced
Calc CHO35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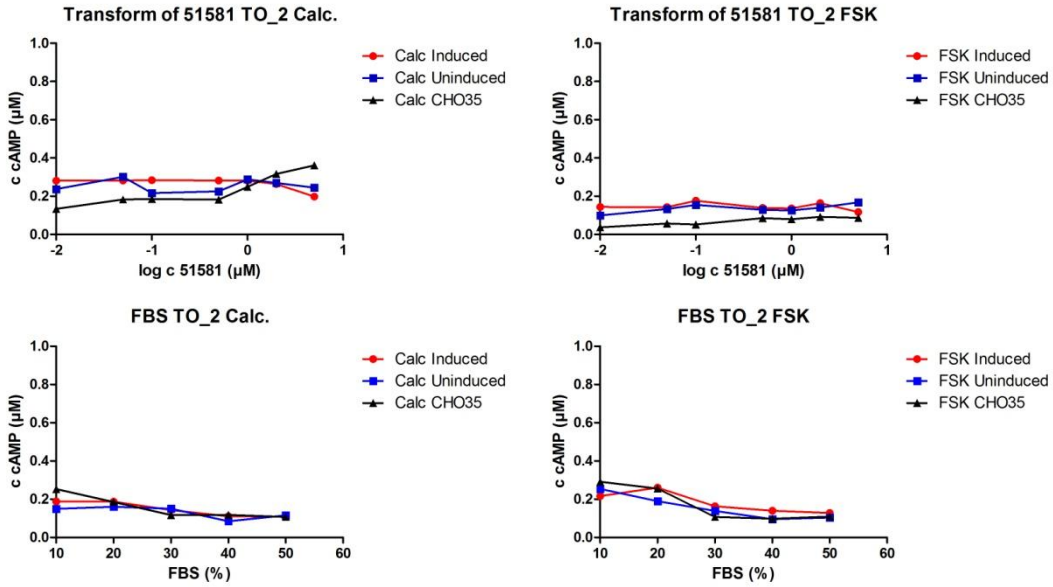
c cAMP (µM)
log c 62966 (µM)
FSK Induced
FSK Uninduced
FSK CHO35



„Pic. 31": Results from cAMP HTRF assay. We reached identical results for all tested clones, thus only results CHO#35 T-Rex TO_2 (clone #2 from conventional cloning) are presented. The number in the graph title always refers to the tested compound according to „Tab. 10", displayed on page 51. X axis represents compound concentration c [µM] in a logarithmic scale (except of FBS [%]), Y axis represents the real cAMP concentration in the cell lysate calculated from cAMP standard curve using GraphPad Prism 5 as an equation of sigmoidal dose-response. Data set was obtained from measurement in triplets. FSK stands for forskolin, Calc. stands for calcitonin. Red line represents response of samples prestimulated with 1 µg/ml tetracycline (24 hours prior the experiment), blue line is the same sample without tetracycline stimulation, black line represents results for parental cell line CHO#35 T-Rex.

3 Summary and discussion

Since 1970 when Dr. Stanfield Rogers performed his first clinical trial, methods of integration into genome made huge progress. In this master thesis I decided to compare three different integration methods using the human G-protein coupled receptor 34 (hGPR34) as our target of interest for integration.

GPR34 was discovered in 1998 by human EST (Expressed Sequence Tags) analysis (Marchese et al., 1999). It is 381-amino acid protein with its gene localized at chromosome Xp11.4 region (Jacobi et al., 2000). Evolution of this receptor started more than 450 million years ago as layed foundations of this receptor can be found in cartilaginous and ray-finned fish genomes (Schulz & Schöneberg, 2003). Structure of GPR34 gene seems to be very high evolutionary conserved among vertebrates (Schöneberg et al., 1999) and no known GPR34-deficient vertebrate has been identified yet (Ritscher et al., 2012).

GPR34 belongs to the group of G-protein coupled receptors with its coupling properties belonging to Gi/o subunit of G-protein (Sugo et al., 2006). Similarities in sequence homology arrange this receptor to the the class I GPCR family (Rhodopsin-like) $P2Y_{12}$-like receptor group. This group includes ADP receptors $P2Y_{12}$ and $P2Y_{13}$, UDP-glucose receptor $P2Y_{14}$ and the orphan receptors GPR87, GPR82 and GPR34 (Marchese et al., 1999). GPR34 also belongs to the group of so-called “orphan” GPCRs, because we do not know any agonist or function for this receptors.

GPR34 exhibits an ubiquitous expression through different tissues (Schöneberg et al., 1999), however most of it is in the cells of immune system like macrophages, microglia and mast cells (Sugo et al., 2006), (Liebscher et al., 2011), (Ritscher et al., 2012). GPR34 expression was also confirmed in lymphoma cells (Ritscher et al., 2012) and cell lines derived from immune system like HL-60 and K562 (Engemaier et al., 2006).

To investigate physiological importance of GPR34, GPR34-knocked out mice were generated. These mice did not show any apparent anatomical or histological abnormalities, changes in body arrangement and development, weight of organs or serum parameters and changes in behavior (Liebscher et al., 2011). However after exposition to a pathogen - *Cryptococcus neoformans,* GPR34-decifient mice showed higher pathogen burden especially in lung, spleen and brain (Liebscher et al., 2011). After immunization with MBSA (Methylated Bovine Serum Albumin) deficient mice showed significantly lower number of granulocytes and macrophages in spleen which indicates that either their migration and/or proliferation are disturbed in GPR34-KO mice (Liebscher et al., 2011). As it results from the experiments of Liebscher et al.,

2011, GPR34 deficiency seems to be compatible with life but its function appears to be tightly bound with successful activation of the immune system.

Several groups already tried to find possible GPR34 agonists (Sugo et al., 2006), (Iwashita et al., 2009), (Liebscher et al., 2011), (Kitamura et al., 2012). The most often mentioned and very controversial substance remains lysophosphatidyl-L-serine (lyso-PS). Lyso-PS is the product of hydrolysis of membrane lipids through phospholipases A1 and A2 after phosphatidylserine exposition to cell surface during apoptosis (Aoki et al., 2002). Due to high GPR34 expression in mast cells and lyso-PS mediated degranulation of mast cells, it was claimed that lyso-PS could be the desired agonist for GPR34 (Sugo et al., 2006). As the results from these experiments give very controversial reports, we decided to include lyso-PS into our experiments as well. However in our results, neither lyso-PS nor any other investigated compound did show any significant activation in cAMP HTRF assay (this was not an HTS).

While finishing this thesis a new paper has been published that might brings a final solution to the lyso-PS/GPR34 paradigm. In an experiment where evolutionary conservation of agonistic specificity was investigated it was found that except for some fish subtypes, no or weak lyso-PS agonistic activity was found among GPR34 orthologues that have been investigated. These data suggest that lyso-PS has an agonistic activity against GPR34 but only on several GPR34 orthologues and additional research is required (Ritscher et al., 2012).

For the generation of hGPR34 stable cell line, we decided to use and compare three different methods of integration into genome: non-directed and random integration using the transfection reagent Lipofectamine™ (Invitrogen), and two novel methods Jump-In TI (Invitrogen) and Meganuclease-driven TI (Cellectis).

Non-directed integration is the most common method being used in laboratories. It allows simple delivery of genetic material into cell. This can be arranged through different mediators e.g. calcium-phosphate precipitation, transfection using cation-lipidic compounds, methods based on physical principles like electroporation or gene gun. Advantage of these methods consists in simplicity and in most cases in affordability. Disadvantage lies in necessity to test many different clones as the integration into genome is only very rare and random and does not take place into the same loci and in inconsistent expression efficiency among obtained clones.

For conventional integration we performed transfection using lipofectamine according to the manufacturers manual. Approximately two weeks post-transfection and

subsequent selection with hygromycin we obtained ten different CHO#35 T-Rex hGPR34 clones. hGPR34 surface expression was verified using FACS analysis and the two best clones (with strongest FLAG-hGPR34 expression) – clone #2 and clone #4 where further studied in functional cAMP HTRF assay.

The Jump-In system which is associated with Gateway cloning consists of two subsequent transfection steps. In the first step we generated so-called platform cell lines (or master cell line) derived from CHO#35 T-Rex cells. Two weeks post-transfection we obtained ten different clones which were tested for the presence (RT-PCR) and number (Southern blot) of integration sites. RT-PCR revealed five succesful clones where integration occurred – clones number #1, #4, #5, #7, #8. Southern blot with these clones prompted us that clone number #1 carries four different integration sites, clones #4 and #7 two integration sites and remaining two clones #5 and #8 one integration site. For subsequent experiments we picked two different clones – clone #1 with four integration sites and clone #4 with two integration sites.

Meanwhile we prepared a so-called expression construct by site-specific recombination between vectors containing our GOI flanked with Flag-tag and CMV inducible promoter. Two recombination steps performed *in vitro* should lead to final construct with a CMV inducible promoter and Flag-hGPR34 sequences assembled in tandem. Simultaneously we performed control recombination reaction where pENTR L1-pLac-LacZα-R5 and pENTR L5-pLac-Spect-L2 vectors were mixed. The resulting expression construct should contain several phenotypic reporter genes to determine the cloning efficiency among them LacZ gene for β-galactosidase expression (MultiSite Gateway Pro manual, Invitrogen). Both Jump-In retargeting reactions (control reaction as well as GOI integration) were performed with a transfection ratio of 1:1 (expression construct : R4 integrase vector) as this ratio gave best results for other GOI´s (data not shown).

Subsequent FACS analysis and control X-Gal staining revealed no Flag-hGPR34 expression as well as β-galactosidase expression. Retargeting experiments were performed two times. Even if we obtained several bacterial clones after transformation which should be due to the presence of different selection markers (kanamycin resistance gene or ampicilin resistance gene) and/or replaceable *ccd*B gene unmistakable, we did not successfully retarget CHO#35 T-Rex Zeo platform cell line. A possible problem could be caused by incorrect integration step catalyzed by R4 integrase and/or LR recombination step between DONR vectors and DEST vector. We previously used the Jump-In Gateway system for a type of glycine receptors which consists of two different functional subunits. We cloned this receptor as one fragment

only with the use of IRES sequence between individual subunits and this receptor was expressed and working correctly. We could support this statement by close analysis of platform cell line where integration sites were confirmed and so that the negative outcome might actually be due to difficulties in Gateway cloning.

Meganuclease-driven TI represents a different approach compared to Jump-In system. The main principle consists in homologous recombination between so-called integration matrix containing GOI and specific sites in genome – in our approach HPRT gene. The integration site is always one, determined by special engineered meganuclease. As it follows, this system is not as versatile as Jump-In (where all mammalian cell lines can be used) and is always designed for a specific cell line. However this system should provide stable fast generated cell line obtained by one co-transfection step only, we observed several difficulties. First, the integration matrix for GOI cloning is quite large (9612 bp); after Flag-hGPR34 cloning our gene of interest had its size enlarged to 12332 bp. It was poorly replicated in *E.Coli* and number of bacterial clones after transformation was very low in comparison with commonly used mammalian expression vectors. This size may also increase the risk of base-exchange during replication. Another problem was long selection phase which reached up to six weeks. Isolated population was growing very slowly and enforced us to lower the antibiotics concentration to half in contrary to those recommended by Cellectis. Despite this fact cell line generated by Meganuclease-driven TI evinced 4 times slower growing in comparison with mother cell line CHO#35 T-Rex. This fact may bring several difficulties in experiments where we compare our cell line with mother cell and where the amount of cells is essential. Despite these facts Meganuclease-driven TI provided us the highest hGPR34 expression (measured by FACS analysis) between investigated samples. Comparison of the integration methods summarizes the table below („Tab. 12”).

„Tab. 12”: Comparison of integration methods being used.

	Conventional transfection	Jump – In TI	Meganuclease - driven TI
Integration into genome	Random	Site specific	Site specific
Cells	All	Mammalian	CHO monoallelic in HGPRT
Characterization of clones	Yes	No	No
Additional needs (except REs, LM, LPF)	No	Platform cell lines, cloning primers, BP/LR enzyme mixtures	Meganuclease expressing vector (cannot be propagated in competent cells)
Selection	3 weeks	2 weeks	6 weeks
Reproducibility	Non-reproducible	Partial (additional characterization of sites in platform cell lines)	Yes
Advantages	Easy, fast, no additional equipment	Specific, stable	Specific, efficient, stable, consistent population, reproducible
Disadvantages	Characterization of clones is necessary (differencies between clones), random integration, generation of pooles, expression can be lost within time	System failed – recombination steps?	Not versatile, specially engineered meganuclease, long selection, cells are growing very slowly (4x), difficult cloning into integration matrix

In the cAMP HTRF assay we tested the agonistic potential of several compounds. These compoucnds were not chosen randomly, their selection was based on selected publications (Chaurio et al., 2009), (Calder & Grimble, 2002), (Guo et al., 2009), (Sugo et al., 2006). Moreover we decided to test the activity of FBS (Fetal bovine serum) after it was claimed to activate GPR34 in another publication (Optimization of Tango™ GPR34-*bla* U2OS cell line; Invitrogen, USA, 2008). Although we had some doubts about performance of this experiment we tried to includes FBS into our experiments as well.

We tested all compounds as refered to in „Tab. 10” with simultaneous stimulation with either forskolin 1 µM forskolin or 100 nm Calcitonin. Unfortunately we did not observe any significant changes in the cAMP levels post-stimulation with any of the compound being investigated.

4 Conclusion

We tried to compare three different *in vitro* approaches for integration of DNA into genome of host cells. As our gene of interest we picked human G-protein coupled receptor 34 – an orphan receptor with no known agonist or physiological function. All approaches were performed and we obtained two differently derived cell lines – from classical random integration and from Meganuclease-driven targeted integration. The Jump-In system which we combined with Gateway cloning failed in our approach, and not only for our GOI (gene-of-interest) but for the positive controls as well. As we already tried this system before with a different cell surface receptor (data not shown), in which cased we cloned both subunits as one fragment using an IRES sequence and did obtained positive results, we suppose that the failure of the system in this approach might be caused by failure in Gateway cloning rather than in Jump-In integration.

In the functional cAMP HTRF assay we did not observe any significant changes and we can say that none of the investigated compounds is a hGPR34 agonist/antagonist. Positive results postulated by Invitrogen (Invitrogen, „Optimization of the Tango™ GPR34-*bla* U2OS Cell Line") may have been caused by poor cell health over the course of the experiment when comparing cells cultivated in the presence and/or absence of fetal bovine serum (FBS). Cells under FBS stimulation were growing normally, compared to the cells cultivated in absence of FBS and the final measurement may be irrelevant as the number of healthy cells in both investigated groups might have been inequal.

We also could not confirm the lyso-PS activation of hGPR34 as postulated by Sugo et al., and we more likely support the statement postulated by Ritscher et al. released in 2012 that the lyso-PS is an agonist only for very specific GPR34 isoforms.

We can say that classical non-directed transfection remains the number one method of choice when investigating unknown genes. Even if we count in the additional amount of work during selection and clone testing, it is still the cheapest, most accessible and most versatile method. We can also recommend Meganuclease-driven targeted integration as we also obtained positive results using this approach.

With this master thesis we also laid the basis for further hGPR34 characterization by generating novel stable cell lines with high surface expression of the receptor. Future high-throughput screening assays could help finding the natural ligand and maybe subsequently the physiological function of hGPR34, thus that the human G-protein coupled receptor 34 may no longer be „an orphan".

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6 Abbreviations

5´UTR	Five prime untranslated region
6-TG	6-thioguanine
AIDS	Acquired immune deficiency syndrome (Acquired immunodeficiency syndrome)
ATP	Adenosine-5'-triphosphate
bp	Base pairs
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGPS	Cellular genome positioning system
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
CNS	Central nervous system
COS-7	CV-1 simian in Origin and carrying SV40
CRE	Cyclic-AMP-response element
CSNB	Congenital stationary night blindness
DAG	1,2-diacylglycerol
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EST	Expressed sequence tags
FBS	Fetal bovine serum
FLIPR	Fluorescent imaging plate reader
FRET	Förster/Fluorescent resonance energy transfer
gDNA	Genomic deoxyribonucleic acid
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
GPCR	G-protein coupled receptor
GPR34	G-protein coupled receptor 34
GRK	G-protein coupled receptor kinase
GTP	Guanosine-5'-triphosphate
hGPR34	Human G-protein coupled receptor 34
HPLC	High-performance liquid chromatography (high-pressure liquid chromatography)
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HTRF	Homogeneous time-resolved fluorescence
HTS	High throughput screening
IFN-γ	Interferon-gamma
IP_3	Inositol 1,4,5-trisphosphate
IRES	Internal ribosome entry site
KO	Knocked-out
LM	Ligation mixture
LP1A	Landing pad
LPF	Lipofectamine
lyso-PS	Lysophosphatidyl-L-serine
MBSA	Methylated bovine serum albumin

MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
O/N	Over night
OFT	Open field test
ORF	Open reading frame
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A (cyclic-AMP-dependent protein kinase)
PKC	Protein kinase C
PLC	Phospholipase C
RAG1	Recombination activating gene 1
RE	Restrictrion endonuclease
RGS	Regulators of G-protein signaling
RNA	Ribonucleic acid
RPMC	Rat peritoneal mast cell
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SPV	Shope papilloma virus
TBP Eu^{3+}	Eu^{3+} trisbipyridine cryptate
$TetO_2$	Tetracycline operator sequences
TetR	Tetracycline-inducible repressor protein
TF	Transcription factor
TI	Targeted integration
TNF-α	Tumor necrosis factor-alpha
T-REx	Tetracycline-regulated expression
WT	Wild-type